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THE INDIAN JOURNAL OF MEDICAL  
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THE  
INDIAN JOURNAL  
OF  
MEDICAL RESEARCH

EDITED BY  
THE DIRECTOR-GENERAL, INDIAN MEDICAL SERVICE,  
THE PUBLIC HEALTH COMMISSIONER WITH THE  
GOVERNMENT OF INDIA  
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THE DIRECTOR OF MEDICAL RESEARCH

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Vol. IX, 1921-22

PUBLISHED FOR  
THE INDIAN RESEARCH FUND ASSOCIATION

BY

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# TABLE OF CONTENTS.

VOL. IX, 1921-22.

## No. 1. (July, 1921.)

	Page
SOPARKAR, M. B. The Cercaria of <i>Schistosomum Spindalis</i> (Montgomery) .. .. .	1
SOPARKAR, M. B. Notes on Some Furcocercous cercariae from Bombay .. .. .	23
CAHUS, J. F. and MHASKAR, K. S. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic Values in connection with the Hookworm Inquiry in the Madras Presidency. No. X—Betanaphthol .. .. .	33
MALONE, R. H. The Production of B. Influenzae Vaccine on a large scale .. .. .	56
MALONE, R. H. A Note on the large scale production of Pneumococcus Vaccine .. .. .	63
HARVEY, W. F. Bacteriological and Laboratory Technique. Section II.—Media .. .. .	66
SINTON, J. A. Some cases of Myiasis in India and Persia, with a Description of the Larvae causing the Lesions .. .. .	132
LANE, CLAYTON. Some Bursate Nematodes from Indian and African Elephants .. .. .	163
HINGSTON, R. W. G. Physiological changes at High Altitudes and their relation to mountain-sickness .. .. .	173

## No. 2. (October 1921.)

CAHUS, J. F., and MHASKAR, K. S. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic Values in connection with the Hookworm Inquiry in Madras Presidency. No. XI—Extractum Filicis, Liquor .. .. .	191
----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------	-----

	Page
CAIUS, J. F. and MHASKAR, K. S. The Correlation between the chemical composition of Anthelmintics and their Therapeutic Values in connection with the Hookworm Inquiry in Madras Presidency. No. XII—Koussou .. ..	198
CAIUS, J. F. and MHASKAR, K. S. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic Values in connection with the Hookworm Inquiry in Madras Presidency. No. XIII—Kamala .. ..	202
CAIUS, J. F. and MHASKAR, K. S. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic Values in connection with the Hookworm Inquiry in Madras Presidency. No. XIV—Pulvis Arecæ .. ..	206
MALONE, R. H. A Bacteriological Investigation of Influenza carried out under the Indian Research Fund Association. Part II—Influenza in Bombay. July, 1919—June, 1920 ..	210
PATON, W. S. Studies on the Flagellates of the Genera Herpetomonas, Crithidia Rhynchodomonas. No. 7—Some Miscellaneous Notes on Insect Flagellates .. ..	230
PATTON, W. S., LA FRENAIS, H. M. and RAO, SUNDARA. Studies on the Flagellates of the Genera Herpetomonas, Crithidia Rhynchodomonas. No. 8—Note on the Behaviour of Herpetomonas Tropica Wright, the Parasite of Cutaneous Herpetomonas (Oriental Sore) in the Bed-bug, Cimex Hemiptera Fabricus .. ..	240
PATTON, W. S., LA FRENAIS, H. M., and RAO, SUNDARA. Studies on the Flagellates of the Genera Herpetomonas, Crithidia Rhynchodomonas. No. 9—Note on the Behaviour of Herpetomonas Donovan, Laveran and Mesnil in the Bed-bug, Cimex Hemiptera Fabricus .. ..	252
ADIE, H. A. A Preliminary Note on the Development of the Leishman-Donovan Parasite in Spleen Juice and in the Alimentary Tract of Cimex Lectularius .. ..	255
HARVEY, W. F. Bacteriological and Laboratory Technique—Section III. .. ..	261
ACTON, H. W. On the Behaviour of Paramecium Caudatum towards the Cinchona Alkaloids .. ..	339
SINTON, J. A. Infection with Nuttallia Ninense among Hedgehogs in the North-West Frontier Province, India ..	359

# Table of Contents.

vii

	Page.
HARVEY, W. F. and IYENGAR, K. R. K. Desiccated Nutrient Media .. .. .	364
IYENGAR, K. R. K. Studies in the Value of the Wassermann Test. No. IV—Significance and Value of a Positive Wassermann Reaction in Tuberculosis .. .. .	369
AWATI, P. R. Some Notes on <i>Conorhinus Rubrofasciatus</i> (de Geer) .. .. .	371
CRAGG, F. W. Geographical Distribution of the Indian Rat Fleas as a factor in the Epidemiology of Plague: Preliminary Observations .. .. .	374
NOTICE .. .. .	399, 401

## No. 3. (January, 1922.)

ADIE, H. A. Telegram announcing finding Leishman-Donovan bodies in Salivary glands and ducts of <i>Cimex rotundatus</i> ..	v
HARVEY, W. F. Bacteriological and Laboratory Technique. Section IV .. .. .	405
CHRISTOPHERS, S. R. and CRAGG, F. W. On the So-called 'Penis' of the Bed-bug ( <i>Cimex lectularius</i> L.) and on the homologies generally of the male and the female Genitalia of this insect .. .. .	415
ACTON, H. W. Melanotic Growths. Researches on the alterations in the Melanoblastic layer of vertebrates, to explain the structure and origin of these growths. Sections I—III .. .. .	464
PATTON, W. S. Some Reflections on the Kala-Azar and Oriental Sore Problems .. .. .	496
CORNWALL, J. W. and LA FRENAYS, H. M. A contribution to the study of Kala-Azar .. .. .	533
CORNWALL, J. W. Note on the Histo-Pathology of a Non-ulcerated Oriental Sore .. .. .	545
PATTON, W. S. Some Notes on Indian Calliphorinas. Part II.— <i>Lucilia Argyricephala</i> Macq. ( <i>Serenissima</i> Fabr.) The Common Indian Bazaar Green Bottle whose Larvæ occasionally cause cutaneous myiasis in animals and <i>Lucilia Craggi</i> Sp. Nov. One of the Common Blow Flies of Indian Hill Stations .. .. .	548

PATTON, W. S. Some Notes on Indian Calliphorinæ. Part III.— Chrysomyia Megacephala Fabr. (Dux Esch). The Common Indian Blue Bottle, whose Larvæ occasionally cause cutaneous myiasis in animals and Chrysomyia Nigriceps Sp. Nov. The Common Blue Bottle of the Nilgiris. . . . .	555
PATTON, W. S. Some Notes on Indian Calliphorinæ. Part IV.— Chrysomyia Albiceps Wied (Putoria Wied Rufifacies Froggatt). One of the Australian Sheep Maggot Flies and Chrysomyia Villeneuvei Sp. Nov. . . . .	561
PATTON, W. S. Some Notes on Indian Calliphorinæ. Part V.— Lucilia Pulchra Wied (Ruficornis Macq.). A Larviparous Calliphorine and Lucilia Ballardii Sp. Nov. A Common South Indian Blow Fly . . . . .	570
SINTON, J. A. Entomological Notes on Field Service in Waziristan	575
HODGKINSON, W. H. and HUTCHINSON, C. M. Report on Electro- lytic Chlorogen E. C. . . . .	586
RAMA KRISHNAN, S. The Reliability of Gaté and Papacostas' Formol-Gel Test for Syphilis as compared with the Wasser- mann Reaction . . . . .	620
SPRAWSON, C. A. The Heart in Beri-Beri and the Evidence of the Differential Stethoscope . . . . .	625
IYENGAR, M. O. T. A Note on grappling tail Hooks in Anopheline Larvæ . . . . .	630

#### No. 4. (April 1922.)

EDITORIAL. Note on bodies found by Mrs. Adie in Salivary glands of Bed-bug . . . . .	v
PATTON, W. S. Some Notes on Indian Calliphorinæ, Part VI.— How to recognise the Indian Myiasis—producing Flies and their Larvæ, together with some notes on how to breed them and study their habits . . . . .	635
PATTON, W. S. Some Notes on Indian Calliphorinæ, Part VII.— Additional cases of Myiasis caused by the Larvæ of Chryso- myia Bezziana Vill, together with some notes on the Diptera which cause myiasis in man and animals . . . . .	654
PATTON, W. S. Notes on Some Indian Aphiochætæ. Aphiochæta Xanthina Speiser (Repicta, Schmitz; Circumsetosa, De Meijere; Ferruginea, Brunetti.) whose larvæ cause cuta- neous and Intestinal Myiasis in man and animals and Aphio- chæta Rufipes, Meigen whose Larvæ occasionally cause cutaneous myiasis in animals . . . . .	683

# Table of Contents.

ix

HARVEY, W. F. Bacteriological and Laboratory Technique— Section V .. .. .	692
HARVEY, W. F. and IYENGAR, K. R. K. Virulence of a Micro- organism and its dependence on the culture medium ..	726
HARVEY, W. F. and IYENGAR, K. R. K. Virulence of the organism as a factor in the efficacy of Prophylactic Vaccines ..	730
HARVEY, W. F. and IYENGAR, K. R. K. On the Immunizing Properties of Allied organisms and non-specific organisms ..	736
HARVEY, W. F. Immunization or Response of Immunized Animals to a small dose of Antigen administered at a long interval after first immunization .. .. .	740
GILL, C. A. Relapsing fever in the Punjab .. .. .	747
MACKEIE, F. P. Visceral Infections due to the Higher Fungi ..	781
CAIUS, J. F. and MHASKAR, K. S. The Correlation between the Chemical Composition of Anthelmintics and their Thera- peutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XV.—Salicylic Acid, and its derivatives .. .. .	787
MHASKAR, K. S. Delayed cures in the treatment of Hookworm Infection .. .. .	799
MALONE, R. H. The Demonstration of the Capsules of the Pneumococcus by a modification of Benian's relief stain ..	804
McVAIL, J. BORLAND. Preliminary Note on Septic tank latrines in relation to Hookworm Disease .. .. .	806
GUPTA, BIRAJ M. DAS. A Note on Some Cultural phases of Leishmania-Donovani .. .. .	809
SHORTT, H. E. Review of the position of Genus Hemocystidium (Castellani and Willey, 1904) with a description of two new species .. .. .	811
SHORTT, H. E. Notes on two Hemogregarines of Persian Lizards .. .. .	827
NAPIER, L. E. A new Serum test for Kala-Azar .. .. .	830
TURKHUD, D. A. and AVARI, C. R. Gate-Papacostas' Reaction in Leprosy .. .. .	850
HARVEY, W. F. and ACTON, H. W. An Examination into the degree of efficacy of anti-rabic treatment .. .. .	852
INSTRUCTIONS TO AUTHORS .. .. .	890
INDEX OF AUTHORS .. .. .	895
INDEX OF SUBJECTS .. .. .	901



# THE CERCARIA OF *SCHISTOSOMUM* *SPINDALIS* (MONTGOMERY).

BY

M. B. SOPARKAR, M.D., Bombay University

*From the Bombay Bacteriological Laboratory*

*With Plates I and II*

[Received for publication October 28, 1920]

CONTENTS.	Page.
Introduction . . . . .	1
Material of Study . . . . .	3
General Description . . . . .	5
Measurements of the Cercaria . . . . .	6
Anal Sucker . . . . .	6
Oral Sucker . . . . .	7
Oral Sucker . . . . .	8
Muscle System . . . . .	8
Excretory System . . . . .	10
Genital System . . . . .	12
Movement . . . . .	13
Internal Anatomy . . . . .	14
Pathology . . . . .	16
Comparative . . . . .	17
Nomenclature . . . . .	19
Summary and Discussion . . . . .	19
References to Literature . . . . .	21
EXPLANATION OF PLATES	

## INTRODUCTION

This cercaria was discovered along with a number of other large-cercous cercariae in Bombay in 1917 in the course of an investigation

## 2     *Cercaria of Schistosomum Spindalis (Montgomery).*

into the possibility of the spread of human Bilharziosis in India. At that time particular attention was being paid to discovering human schistosome cercariae in this country, and the finding of one which possessed the main characters of these cercariae aroused considerable interest. At first it appeared that the new form found in Bombay was probably one of the human cercariae, but as no more detailed description of their structure beyond the main features given by Leiper was at that time available, identity of the present species with the human form could not be taken as established on superficial morphological similarity alone. It was, therefore, proposed to settle this point experimentally by attempting to raise adult parasites from the cercariae, and comparing the structure of the mature adults with that of the schistosomes already known. For this after all is the only final test of the identity or otherwise of the larvæ with the adult. Structural correlation between a known cercaria and one under investigation may at best be suggestive of their possible identity, but the final proof must rest with the experimental rearing from them of identical adults.

Of course, the experimental method too has many pitfalls and unless great care is taken the results are apt to be misleading. Indeed, as Cort<sup>(1)</sup> observes 'in some very recent work, as well as in older papers, larvæ and adults, shown later to be entirely unrelated, have been joined experimentally.' But this only emphasises the great importance of care and proper controls in conducting such experiments.

It was deemed advisable not to give the cercariae any specific names—the cercaria being but a larva of the adult parasite—but to give them provisional numbers till such time as their life-history had been worked out, when they would naturally be named after the adults. Accordingly this cercaria was designated *Cercaria bombagensis* No. 4.

Infection experiments were carried out which proved that this cercaria was not of a schistosome which affects human beings, but was of an animal infecting type—*Schistosomum spindalis*. A brief account of these experiments has already been published in the *Indian Journal of Medical Research*.<sup>(2)</sup> The results have since been confirmed by repeated experiments, and there is now no doubt that this cercaria forms the larval stage of *Schistosomum spindalis* (Montgomery). Life-history studies on furcocercous cercariae have been very meagre and this appears to be the first instance in which an animal schistosome cercaria has been discovered and its life-history traced through the various stages of its

development. The present paper will be concerned with a description of this cercaria and it is hoped that it will enable workers to find new endemic places of the disease. Indeed this has been the case in Bombay, for the occurrence there of this cercaria indicated the local existence of the disease and when animals were examined cases of actual infection with this parasite were found in cattle. The first case in this part of India (Bombay) was found in a cow in February 1918. This is significant, for the cercaria has been recorded but once by Vixberg<sup>1</sup> from Sumatra since the discovery of it by Montgomery<sup>2</sup> about fourteen years ago, in two cattle in Northern India. The knowledge of the structure of the cercaria will reveal many new endemic areas of this disease. And for the discovery of the existence of animal schistosomiasis in any locality, examination of the local snails for specific cercariae is likely to be found more convenient and easier to carry out than a systematic examination of carcasses of several animals, especially in places where there are no regular abattoirs.

#### METHOD OF STUDY.

The method adopted for obtaining cercariae is somewhat similar to one of the two methods used by Leiper<sup>3</sup> in his investigations in Egypt. Snails are collected on the previous day in lots of 150-200, cleansed of all adherent dirt, and distributed with a small quantity of clean tap water in test tubes, each containing a single specimen, which are left overnight. Next morning the water in the test tube is examined with a hand lens for the presence of cercariae. Experience has shown that over ninety per cent. of the infected snails in which the cercariae have matured discharge them into water within twenty-four hours. The cercariae are shot out by the snail in puffs containing fifty or more individuals and this action appears to be stimulated by contact with fresh tap water. In several instances when an infected snail containing mature cercariae was placed in a test tube and fresh tap water was added, the snail was observed to discharge cercariae into water within a few minutes. These cercariae would be available for study for about twenty-four hours only; for the life of these larvæ in a free state is rarely longer than this; but as successive batches of mature individuals would be discharged by the mollusc at intervals, a continuous supply of these forms can be obtained by this method for study or for experimental purposes, as long as the mollusc survives. As a rule the life of an infected mollusc is much shorter than that of a non-infected one, those in which the

#### 4     *Cercaria of Schistosomum Spindalis (Montgomery).*

cercariae have matured were observed to die within two to six days after being captured; occasionally, however, they have been known to live for some weeks.

When the study of the mature forms is completed or in the meantime if the snail shows signs of approaching death, often indicated by the red tinging of the water with the blood of the mollusc (*Planorbis*), the animal is dissected for further study of the infected organs, parthenitæ, etc. This method is preferable to a systematic dissection of all the specimens collected, a much more laborious task and one in which every snail has to be sacrificed. Moreover when an infected individual is found, an incident which may not happen for several days, the study has to be complete during the rest of the day—which is not often possible—for the cercariae and the sporocysts will hardly remain alive for more than a day. The only advantage systematic dissection has over the above method is that it reveals immature infection also which the latter does not; an advantage of importance when the percentage of infected snails in a locality is to be ascertained.

A satisfactory study of the structure of these forms can only be made in living specimens; and for tracing the finer branches of the excretory system and other details an oil immersion lens with a good resolving power is indispensable. For studying the flame cells and their ducts, artificial light was found more suitable.

In the course of this study the method of intravital staining with dilute solution of neutral red was employed in several instances. By this method it was possible to slow down the activity and the vibration of the cercaria sufficiently to allow of the study of its morphology without exerting undue pressure which is often required to keep it steady. It has another advantage of bringing into relief certain structures and their outlines which by their transparency appear indefinite. Cort<sup>(6)</sup> did not find any advantage in the method of intravital staining in the study of the cercaria of *Schistosomum japonicum*. While it may be granted, that in the case of certain cercariae in which the structures are clearly differentiated, this method may not offer any advantage, nevertheless, it cannot be doubted that by this method the activity of the cercaria is inhibited and its study thus facilitated especially when the activity is great and the study on this account rendered otherwise difficult as has been found by Miyagawa and other Japanese observers. Sections of the infected liver were also studied to confirm or to check observations made upon living specimens,



# PLATE I.

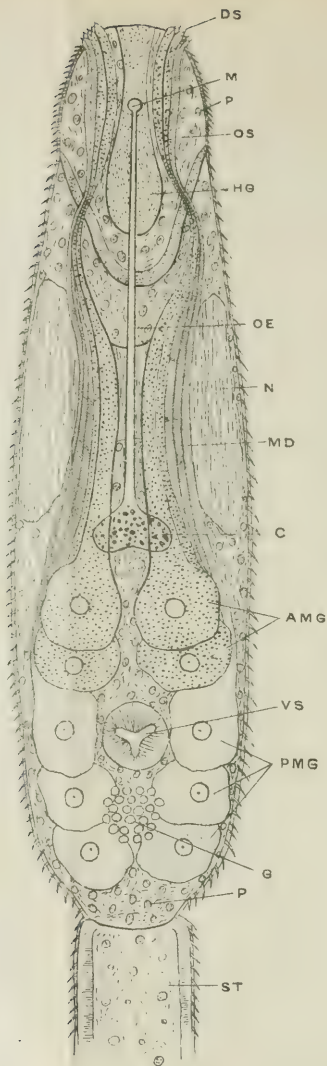


FIG. 2.

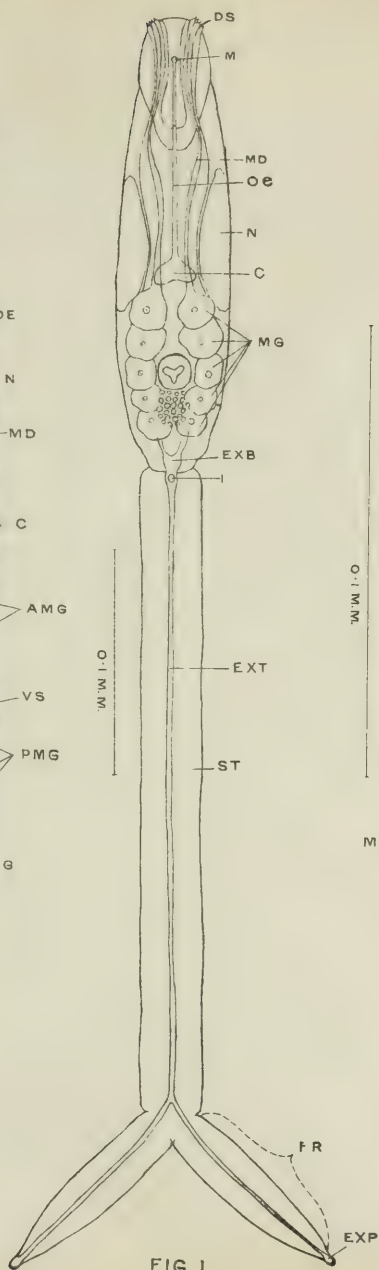


FIG. 1.

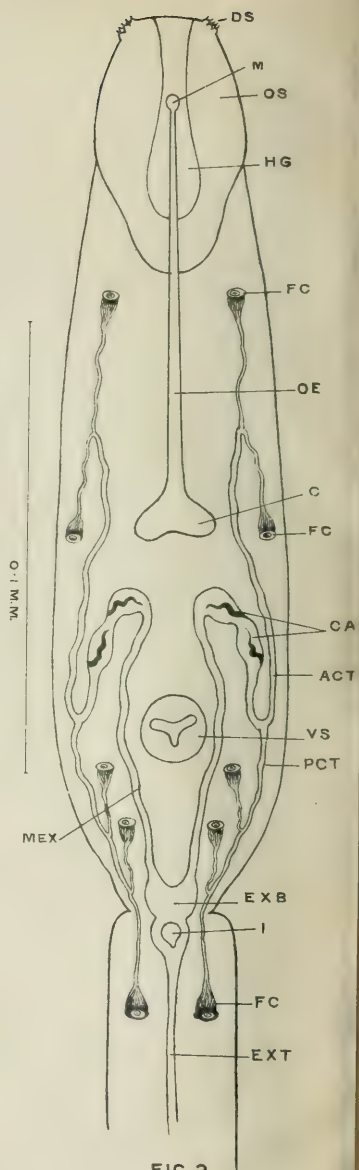


FIG. 3.

# DESCRIPTION OF PLATE I.

- Fig. 1. General view of the whole animal, showing the position of the different structures and the excretory organs.
- Fig. 2. Showing details of the internal organs.
- Fig. 3. Showing details of the excretory organs.

DESCRIPTION OF PLATE I.

- Fig. 1. Cercaria of *Schistosomum pendalis*: general view of the whole animal with outlines of different structures and the excretory tubule in the tail.
2. Cercaria of *S. hesiosomum speculalis*, showing details of the internal structure: ventral view.
3. Cercaria of *Schistosomum speculalis*, showing details of the excretory system (semi-diagrammatic).

## GENERAL DESCRIPTION.

This cercaria belongs to the furcocercous group of distance cercariae possessing a body provided with an oral and a ventral sucker, and a tail which is bifid at its distal end (see Plate I, Fig. 1). The body including the entire portion of the head except a small central part, represented, as will be seen later, by the opening of the head gland, is covered with sharp backward pointing cuticular spines. These are especially marked over the head. The tail and the furcal rami are also provided with spines, but they are somewhat sparse in this region. The anterior end, called the head, is provided with an invertible pouch or a protrusible snout which appears to be in constant action.

The body possesses great powers of extension and retraction due to the presence under the cuticula of layers of muscle fibres; the fully extended body often measuring twice as much as during contraction.

The tail and the furci are only larval appendages and their morphology is comparatively simple.

The proximal end of the tail is somewhat convex and fits into the small concavity surrounded by a slight thickening in the cuticula in the posterior end of the body. At its distal end are two bifurcations—the furcal rami—which are distinctly constricted off from it. The tail is round in cross section while the furci are flat. The former contains several cells with clear cytoplasm and large nuclei distributed in the parenchymatous tissue. Their number varies from 150—175 or sometimes more, and are well brought out by the method of intravital staining. The distribution of the nucleated cells is continued in the furci and in this situation their number varies from 30—35. The excretory duct runs throughout the length of the tail and the furcal rami.

The important part of the cercaria is the body which eventually develops into the adult parasite, the tail being dropped prior to entrance of the larva into the body of the final host.

Besides the oral and the ventral suckers, the body contains a group of what may be termed mucin glands. The ducts from these glands enter the oral sucker and thus open externally. It also contains alimentary, nervous, genital, and the excretory systems.

## STRUCTURE OF THE CERCARIA.

*Measurements.*—There is a tendency to depreciate the value of measurements and to regard them as unsatisfactory data for comparison.

of different cercariæ owing to the great power of extension and retraction not only of the body, but also to some extent of the tail and even the furci possessed by these larvae. This is no doubt true in a general way: and it is also true that measurements of preserved specimens will vary with the preservative used and the state of contraction at the time of fixing. But by adopting a method of killing by heat, used by Bahr(?) it is possible to take measurements which will allow of satisfactory comparison being made. It has been found during this investigation that if cercariæ are killed by gentle heat, the measurements of such specimens are more uniform than those treated by other methods of killing, and this method has the further advantage of being simple and generally applicable so that measurements taken by different observers by this method can be rendered comparable. The method employed is as follows:—

Place a large drop containing several cercariæ on a glass slide and gently heat it by holding it high over a bunsen flame taking care not to allow the drop to boil. Examine at intervals of a few seconds under a low power microscope until most of the cercariæ are found to be motionless—put on a coverslip taking care that it does not exert any pressure on the cercariæ and proceed to take measurements.

The following are the average measurements of specimens examined by the above method:—

Body—200  $\mu$  long by 50  $\mu$  broad; variation in length 180  $\mu$ —220  $\mu$

Tail trunk—290  $\mu$  long by 30  $\mu$  thick: variation in length 270  $\mu$ —300  $\mu$

Furcal Rami—100  $\mu$  long, variation in length 90  $\mu$ —120  $\mu$

In living specimens the body when fully extended measures 250  $\mu$  long by 35  $\mu$  broad, while during greatest contraction it measures only 125  $\mu$  long, but the thickness increases to 70  $\mu$

Below are measurements of specimens preserved in 5 per cent. formalin, which are smaller than those killed by heat:—

Body—150  $\mu$   $\times$  50  $\mu$ : variation in length 115  $\mu$ —180  $\mu$

Tail trunk 275  $\mu$   $\times$  30  $\mu$ ;      do      do      do 250  $\mu$ —300  $\mu$

Furcal Rami 90  $\mu$       :      do      do      do 75  $\mu$ —100  $\mu$

*Ventral Sucker.*—The ventral sucker is a strong muscular structure situated about four-fifths of the body distance from the anterior end. It is circular in outline with a triangular opening and has a diameter of .02 mm. Its surface is covered with sharp cuticular spines which are more numerous in this situation than on the surface of the body in the immediate neighbourhood. It serves an important part in



# PLATE II.

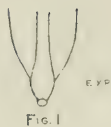


Fig. 1

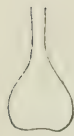


Fig 2



Fig 3

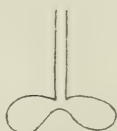


Fig 4

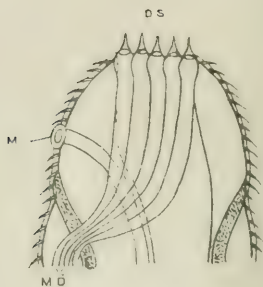


Fig.5

Sch.  
HEMATOBIUM



Sch.  
MANSONI



Sch.  
JAPONICUM.



CERCARIAE  
INDICAE.  
TYPEXXX.  
(CAPT SEWELL)



Sch.  
SPINDALIS



Fig 6 X 100.

## DESCRIPTION OF PLATE II

- Fig. 1. Lateral view of the excrescence showing the opening of the excrescence from the side of the head.
- Fig. 2. Lateral view of the excrescence showing the different shapes of the excrescence.
- Fig. 3. Lateral view of the excrescence showing the shape of the head of the excrescence with its basal part and the shape of the excrescence.
- Fig. 4. Lateral view of the excrescence showing the shape of the excrescence magnified to the same extent.

DESCRIPTION OF PLATE II.

- Fig. 1. Cercaria of *Schistosomum spindale*, opening of the excretory pore at the tip of furcal rami.
- Figs. 2, 3, 4. Cercaria of *Schistosomum spindale*: different shapes of the cæcum.
- Fig. 5. Cercaria of *Schistosomum spindale*. Side view of the head showing mouth with its muscular sphincter and the spined ends of mucin ducts.
6. Outlines of the various schistosome cercariae magnified to the same extent.

hemostation. Though a prominent structure, it does not project beyond the surface to such an extent as to appear as a proboscis as is the case with the Japanese form.

*Digestive System.*—The digestive system is simple and consists of a mouth, an oesophagus, and a kidney-shaped caecum. The mouth opens on the ventral surface not at the extreme anterior tip of the body but a short distance behind it at a point a little in front of the constriction which marks off the head from the body. (See Plate I, Fig 2, also Plate II, Fig 5.) When viewed from the side the opening of the mouth is seen to be surrounded by a knob-like umbilicated thickening which probably represents a muscular sphincter around the mouth (See Plate II, Fig 5). Neither in his description of the cercaria of *Sch. japonicum*, nor in his figure does Cort<sup>(6)</sup> show the structure, while in the figure of *C. unicolor* type XXX, described by Captain Sewell<sup>(7)</sup> as resembling the Japanese species, the opening of the mouth is shown a little further forwards and there is also no mention made of the muscular sphincter. A narrow buccal cavity leads to an oesophagus which passes caudad and dorsad extending to a little behind the middle of the body where it opens into a kidney-shaped vesicular structure—the caecum.

There is no indication of the presence of any muscular pharynx.

The caecum is a conspicuous structure, containing granular material, and is brought out especially prominently by the method of intravital staining when it appears as a large clear vesicle containing stained granules which are constantly agitated by its contractile movements. Even after cessation of these movements the smaller granules will continue to show brownian movements. The caecum is surrounded posteriorly by mucin glands and lies dorsad to the ducts of these glands. Constriction of the organ in the middle sometimes divides it into two chambers connected by a narrow isthmus and the peristaltic movement of the organ causes the contents to flow from one chamber to the other. The shape of the caecum varies greatly: usually it is kidney shaped, sometimes it is heart shaped, and occasionally dumb-bell shaped, depending upon the pulling up of the oesophagus and the degree of constriction in the middle. (Figs. 2, 3, 4 in Plate II show the various shapes of the caecum.) This structure is present in the caecum of *Sch. japonicum*, but no mention of it is made by Captain Sewell<sup>(7)</sup> in his description of the new cercaria stated to resemble the former. The structure itself is so prominent that it

cannot easily be missed, and the absence of it in Captain Sewell's specimen is remarkable.

*Oral Sucker.*—The oral sucker is a thick muscular structure situated at the anterior extremity of the body. It measures  $64\ \mu$  in length and  $15\ \mu$  at its broadest part. It may be divided into an anterior and a posterior portion. The anterior portion is in direct contact with the cuticle of the body—its wall in this situation being reinforced by muscular fibres under it, while the posterior portion is separated from it by paranchymatous tissue. This portion is pyriform in shape and very muscular. Its wall contains layers of longitudinal and circular muscle fibres and has a thickness of  $\cdot 008$  mm.

In the centre of the oral sucker is situated what is called the head gland. This gland is granular and opens externally in the middle of the head between the two groups of mucin ducts—(See Plate I, Fig. 2). It is surrounded by several nuclei, but these do not seem to be a part of the gland. The œsophagus pierces the posterior portion of the oral sucker in the middle line while the mucin ducts do so on either side of it. The position of the mouth in the oral sucker has already been described. The rest of the oral sucker is filled with paranchymatous tissue and nuclei. The relation of these various structures in the oral sucker is shown in Plate I, Fig. 2.

The anterior end of the body is provided with what appears to be a protrusible snout. In reality it is a part of the anterior portion of the oral sucker which is rhythmically pushed out and rolled in by the strong muscular action of the posterior portion. When the snout is rolled in, the opening of the head gland as well as those of the mucin ducts on either side of it are turned towards the centre of the sucker, but when it is pushed out the spined ends of the mucin ducts are turned outwards and thus come in contact with any object to which the head is applied; at the same time the muscular action of the posterior portion of the oral sucker forces out the contents of the ducts through their spined ends. This movement of the anterior end of the body has correlation with penetration of the cercaria into the final host. This subject will be discussed later.

*Mucin Glands.*—The posterior half of the body of this cercaria is filled with a number of unicellular glands disposed around the acetabulum. One could make out five such glands on each side of the middle line. They may be divided into two groups—an anterior and a posterior one. The anterior group consists of two pairs of flask-shaped glands measuring

about  $0.1 \times 0.15$  m.m. with rounded basophilic nuclei and is situated in front of the ventral sucker. The protoplasm of this group of glands is coarsely granular and the granules are acidophilic. In stained sections the granules take a deep eosin stain like the granules of eosinophile leucocytes and stand out prominently. The posterior group consists of three pairs of glands and is situated on either side and behind the acetabulum. These glands contain slightly larger nuclei and their protoplasm is very finely granular or almost homogeneous. In stained sections it takes a faint hematoxylin colour and stands in marked contrast with the large acidophyle granules of the anterior group. This contrast is also brought out in the living cercaria stained by the intravital method when the anterior group of coarsely granular glands remains unstained while the posterior group appears deeply stained.

The differentiation between the coarsely granular acidophilic and the finely granular or almost homogeneous basophilic group of glands has also been noted in the cercaria of *Sch. mansoni* (Faust)<sup>(8)</sup> and in the *Ceratomyxidea* type XXX, described by Captain Sewell<sup>(9)</sup>, though in the latter Captain Sewell describes both groups as acidophilic. It is worthy of note that this differentiation is not found to exist in the cercaria of *Sch. karnatakensis* and *Sch. japonicum*—(Faust)<sup>(10)</sup> Cori<sup>(11)</sup>.

From these glands ducts pass forwards one from each gland along either side of the middle line to enter the oral sucker and open at the anterior end. Ducts from each group of glands can be distinguished by their contents which are coarsely granular in one case and almost transparent in the other. In the middle of the body the course of these ducts is deflected towards the middle line in order apparently to accommodate two large masses—the lobes of the central nervous system—between them and the body wall on either side. These two lobes appear to be connected by a median commissure lying dorsal to the ducts. No attempt was made to trace the branches of these lobes. At the upper border of these lobes the ducts are again deflected outwards when they enter the oral sucker. The ducts at the entrance are considerably constricted while passing through the thick muscular wall of the sucker. (See Plate II, Fig. 5). Within the sucker they again spread out and open externally at the anterior end of the protrusible snout on each side of the middle line. Near the opening the ducts are slightly expanded giving the ends the appearance of knobs, and are capped by hollow piercing spines. It is rather difficult to make out the exact number of these spines, for in a living preparation they do not all lie in the same plane, but in a sort of a

## 16 *Cercaria of Schistosomum Spindalis* (Montgomery).

bunch and cannot all be brought into focus at the same time : and the constant pushing out and rolling in action of the snout often makes this determination extremely tedious. The relation of the hollow spines to the ducts has not been clearly shown by Cort. In his description of the cercaria of *Sch. japonicum* and also in his figure (Cort<sup>(6)</sup>, page 493, Fig. 1) he has shown five spines on one side and only four on the other. although the mucin glands have been stated to be five on each side. It must be clearly understood that the relative number of mucin glands, mucin ducts and the hollow spines is always the same, for it has been determined that a single duct arises from each mucin gland and that each such duct is capped by a hollow spine, and it is through these spined ends of the ducts that the secretion of the mucin glands is ejected.

This is true not only of this species but also of other furcocercous cercariæ examined by the writer. These observations entirely agree with those of Faust made on cercariæ of *Sch. hematobium*, *Sch. mansoni* and other schistosome larvæ, and indicate the probable function of these glands, which appears to be to supply the secretion which by its cytolytic action may help the cercaria to create a breach of surface against which it applies its head with the spined ends of ducts and thus facilitate penetration into the body of the host.

*Excretory System.*—The excretory system of this cercaria is typical of the schistosome group. It closely resembles that in the cercaria of *Schistosomum japonicum* in its general arrangement, and in the course and distribution of the main excretory trunks, though it differs from the latter chiefly in the number and position of the flame cells. The main excretory trunks in the body arise as the anterior continuations of the two cornua of the bladder (see Plate I, Fig. 3), and taking a somewhat tortuous course pass forwards on each side of the acetabulum to a point midway between it and the cæcum. At this point each trunk makes a bend and runs outwards and backwards for a short distance, when at the level of the acetabulum it divides into two branches, the anterior and the posterior collecting tubules. In the portion of the main trunk between the beginning of the bend and its bifurcation, are two fusiform dilatations each containing a bunch of vibratile cilia. These are attached to the inner wall of the tube and appear to serve the purpose of driving the contents of the tortuous tube in which the flow is liable to stagnate. Indeed in specimens stained by the intravital method small excretory granules were observed to be swept by their current and were noticed to enter the bladder.

While examining living specimens which are only partially flattened several bright points are seen in the course of the main trunk. These are due to numerous kinks produced by pressure upon the tortuous tube.

The anterior collecting tubule passes forwards (cephalad), and is joined by two smaller tubules arising from the flame cells situated in front of the acetabulum. The most anterior of them is located a little below the lower border of the oral sucker about midway between the middle line and the outer border of the body. Its cilia are directed backwards (caudad), and a long thin tubule arising from this passes backward where it is joined a little in front of the cæcum, by another short tubule from the second flame cell located at the level of the organ close to the outer border of the body. Cilia of this flame cell are directed inwards and forwards and the tubule runs in the same direction.

The posterior collecting tube passes backwards (caudad), and is joined by smaller tubules arising from three flame cells situated as follows:—

One in the tail about 0.5 mm. from its proximal end. The other two are situated in the posterior part of the body behind the acetabulum. The position of the flame cells and the distribution of the excretory tubules is shown in Plate I, Fig. 3. The flame cells in the tail are somewhat larger than those in the body. The action of their cilia appeared to be rendered more vigorous by slight pressure. In more than one instance they were observed to keep on flickering for over a minute after the detachment of the tail.

The excretory bladder is situated at the posterior end of the body. It is thin walled and bicominate in shape and is continued backward where it is joined by the tubule in the tail. The junction is marked by a characteristic anastomosis which gives rise to the formation of an island and gives an indication of the original paired arrangement of the tube in the tail. The excretory duct in the tail runs as a single tubule to its distal end where it bifurcates to enter the forestoma and passes to the tip where it opens externally in a peculiar bulbous expansion. (See Plate I, Fig. 1; also Plate II, Fig. 1.)

Cort has drawn attention to the importance of the excretory system as a means of distinguishing schistocephalæ cercariæ. In his paper on the

Homologies of the excretory system of the forestal (tail) cercariæ, he has shown how markedly the number and position of the flame cells differ in different furcocercous cercariæ.

## 12 *Cercaria of Schistosomum Spindalis (Montgomery).*

Comparing the present species with the cercaria of *Sch. japonicum* one finds several points of agreement, *viz.*, the course and arrangement of the excretory tubules both in the body and in the tail, the two ciliated areas, the bladder, the 'island,' and the two excretory pores at the tip of the furci with their bulbous ends. Two points of difference will, however, be noted. The character of the ciliated areas in the expanded portion of the main trunk in the body is different (*vide* Cort<sup>(6)</sup> *pl. g.* 497, Fig. 2), and that there are four pairs of flame cells in the body of this species, while the Japanese species possesses only three.

It must be stated here that this system is so difficult to study and to trace with accuracy that it is little wonder that different observers gave incomplete and varying description of it, *e. g.*, Miyairi and Suzuki<sup>(11)</sup> stated that the excretory system of the cercaria of *Sch. japonicum* has five pairs of flame cells. Miyagawa (quoted by Cort)<sup>(6)</sup> saw only the main tubules of this system in the body and missed its connections in the tail. Ogata<sup>(12)</sup> found two paired laterally symmetrical flame cells with vessels. While Cort has definitely stated that it possesses three pairs of flame cells in the body and one in the tail. Again Iturbe<sup>(13)</sup> saw five pairs of flame cells in the cercaria of *Sch. mansoni* including one pair in the tail—a number identical with that in the present species, but gives no description of the details of the system; and Manson-Bahr and Fairley<sup>(14)</sup> state that the excretory system of these schistosome cercariæ (*Sch. hæmatobium* and *Sch. mansoni*) 'consists of six pairs of flame cells arranged along the margins of the body.' Cort, however, from his knowledge of the homologies of the excretory system of the forked tailed cercariæ in general would expect to find this system in the cercariæ of *Sch. hæmatobium* and *Sch. mansoni* closely corresponding to the conditions described by him for the cercaria of *Sch. japonicum*.

*Genital System.*—The genital system of this cercaria is rudimentary and is represented by a cluster of small round cells situated ventrally just posterior to the acetabulum. One could count about twenty-four cells in such a cluster. No collections of cells representing the testes described by Captain Sewell<sup>(8)</sup> for the *cercaria indica*, type XXX, could be made out, though several paranchymatous cells found in other regions of the body were also present on the posterior and lateral border of the body.

The determination of the sex of the schistosome cercariæ is a matter of considerable difficulty; in fact, it is not possible, in the present state of

our knowledge, to say with any certainty whether a particular cercaria will develop into a male or a female schistosome, nor is it definitely known whether these cercariae are, as is suggested by Captain Sewell, and also by Faust<sup>(15)</sup> by his description of *Cercaria gracillima*, hermaphrodite, and during development one of the sex elements present is suppressed or that even in the larval stage the rudiments of the genital system are specific, and that the male cercaria develops into a male schistosome and a female into a female adult. Lepper<sup>(16)</sup> suggests that the cercariae from a single miracidium may all be of one sex and states that he tried to avoid getting adults of one sex only in his experimental animals by using cercariae derived from more than one specimen. Some experiments were made to test this point. Young goats were subjected to infection with the cercariae derived from a single mollusc. The number of cercariae obtained varied from 10,000 to 15,000. After the lapse of about eight weeks, the animals were examined, but in no case were any mature parasites found in them. The result may have been due to the paucity of the cercariae employed, for in the case of other similar experiments in which mature schistosomes of both sexes were found as many as over 100,000 cercariae derived from several molluscs were employed.

**Movement.**—When observed in a small quantity of water in a test tube the cercariae can just be seen with the naked eye. With a hand lens they are seen to hang in water with the head down and the tail turned upwards. In this position the cercariae do not remain stationary, but gradually sink when suddenly they begin to swim actively and in this action rise to a certain height in the water again. This movement is in a more or less straight line either perpendicularly upwards (and this is the most usual direction) or at an angle—but the motion is never of an irregularly winding character as in some other furcocercous cercariae found in Bombay.

On a substratum the cercaria moves by a rapid crawling movement. The exact manner may thus be described. The animal fixes itself to the surface by means of its ventral sucker, the fins of the tail come together and both the body and the tail then undergo rapid vibration, the ventral sucker acting as a pivot. After a second or two, the vibration ceases, the fins open out, the portion of the body in front of the ventral sucker is extended, and the oral sucker takes hold as far forward as possible. The hold of the ventral sucker is then released, the body is contracted and arched, the ventral sucker is thus pulled backwards and takes a fresh hold. The hold of the oral sucker is then let off and the body and tail

#### 14 *Cercaria of Schistosomum Spindalis (Montgomery).*

rapidly vibrate when the whole manoeuvre is repeated and the cercaria makes rapid progress. After some time the animal appears tired and the speed of the movement is slowed down. During this slower progress the cercaria bends its body and appears to be in the act of licking or biting at the ventral sucker which is projected sideways every time it makes a forward move. After some time the cercaria sheds its tail which keeps on vibrating for some time, and the body moves in a slow creeping sort of movement and eventually the larva dies.

If, however, on its way the cercaria meets with some soft obstruction such as debris or snail tissue its movements often acquire a vigour and persistence so that the cercaria finally succeeds in penetrating the tissue. These movements appear to have a correlation with penetration into the skin of the host. In several instances cercariæ were watched in a mass of debris and snail tissue. The movements executed resembled intelligent attempts at penetration. A firm hold was obtained by means of the ventral sucker and the body extended and reached in all directions as if trying to find a suitable point. Having found one it applied its anterior end against it and vigorously pushed out the protrusible snout. This pushing out of the snout turned the spine pointed ends of the gland ducts outwards and brought them in direct contact with the tissue. Several times during such movements minute droplets of secretion were observed to issue from the ends of the ducts. The snout would then be rolled in and by this alternate pushing out and rolling in action the cercaria would effect a breach in the surface, and the anterior end would be pushed in by the extension of the body. The spines on this portion of the body would prevent it from slipping back when the hold of the ventral sucker is released, and by taking a fresh hold in advance of the previous one and repeating the pushing movements the cercaria would succeed in penetrating the tissue.

These observations give an idea of how the cercaria enters the host, for it is chiefly by penetrating the skin that infection by this parasite takes place as has been shown by successful artificial infection experiments by this method (*vide* Liston and Soparkar).<sup>(2)</sup> Faust found no record of encystment in the group of furcocercaria and this species is no exception to the rule.

*Intermediate host.*—The cercaria of *Sch. spindalis* develops in *Planorbis exustus* and was first found in January, 1917, in sloughs in Mahim, near Bombay. The mollusc itself is widely distributed. The

percentage of infection shows a seasonal variation (as will be seen from the following Table I).

TABLE I  
*Showing seasonal variation in Molluscan infection.*

Month	No. of snails examined	Percentage found infected with the present parasite	Month	No. of snails examined	Percentage found infected with the present parasite
1917			1918		
January	376	0.03	January	2,129	0.11
February	64	—	February	1,674	0.04
March	229	—	March	1,804	0.03
April	106	—	April	2,111	0.09
May	417	0.04	May	372	1.07
June	419	0.08	June	1,166	2.06
July	1,242	0.48	July	1,442	4.3
August	781	0.89	August	2,150	1.76
September	1,052	8.6	September ..	1,753	6.92
October	1,792	3.9	October ..	367	3.3
November	1,544	0.91	November ..	288	0.3
December	1,214	0.06	December ..	423	0.06

Note.—The snails were examined for infection by looking for necrosis due mainly to *Myxosporidium*.

It will be noted that the incidence of infection is highest during the autumn months and lowest during late winter and early spring. In a majority of instances the molluscs were infected with a single species, but in some instances there was a double infection, the species being associated with another *Xytrichocerca* or with *Tricostema* and on one occasion, all three types were found in a single mollusc. Rarely this form has been noted in *Lymnaea stagnalis*.

## 16 *Cercaria of Schistosomum Spindalis (Montgomery.)*

Specimens from this appeared morphologically identical with the cercaria of *Sch. spindalis*, obtained from *Planorbis*, but attempts to infect animals with these did not prove successful. The failure may partly be attributed to the small number of cercariæ obtained.

*Parthenitæ*.—The infected organ of the snail has sometimes a yellowish brown and at others a dark slate grey colour. The cercariæ develop in small cylindrical sporocysts measuring about 1.2 mm. in length and about .15 mm. in diameter. The wall is very thin and is formed by a delicate layer of connective tissue lined by a single layer of nuclei. It is, however, elastic and allows of considerable stretching by the movements of the contained cercariæ. Each sporocyst contains from 6 to 8 cercaria in various stages of development. The dark colour of the infected organ is often due to the deposition of numerous dark granules in the wall of the sporocyst. The sporocysts are firmly attached to tissues of the digestive gland, and it is difficult to tease out separate individuals. No rediæ formation has been noticed in this species.

Cort<sup>(6)</sup> states that the cercariæ of the human schistosomes are very small and develop in elongate *motile* sporocysts (*italics mine*). The cercariæ of *Schistosomum spindalis* as mentioned above also develop in small elongate sporocysts but the latter are *not motile*. The writer has during this investigation met with other furcocercous cercariæ which develop in elongate motile sporocysts, but these have not yet been shown to belong to the group of schistosomes.

*Comparison*.—In the accompanying Table II partly adapted from Faust a comparison is made between the present species and the human species of schistosome cercariæ and also the form recently described by Captain Sewell.

The features for comparison given in this table are more detailed than those given by Leiper who characterised *Sch. cercariæ* as apharyngeal furcocercous cercariæ without eye-spots. Much work has, however, been done by Iturbe, Gonzalez, Cort, Faust, and others since Leiper's original description of these forms, and several furcocercous and other cercariæ have recently been studied and described. In view of the above, it would be advisable to define in greater detail the limits of the human and animal schistosome cercariæ.

An attempt is made in the following tentative table to present the main anatomical and other features which are common to all the human

TABLE III

[illegible]

and animal forms so far studied leaving aside the consideration of *Gigantobilharzia* (Odhner<sup>17</sup>), *Austrobilharzia* (Johnston<sup>18</sup>), *Ornithobilharzia* (Odhner<sup>19</sup>) and other avian forms, of the cercariæ of which we have at present very little knowledge.

TABLE GIVING SOME COMMON CHARACTERS OF KNOWN HUMAN AND ANIMAL SCHISTOSOME CERCARIÆ.

1. They are distomes—having an oral and a ventral sucker.
2. They are furcocercous cercariæ—having a bifid-tail.
3. The furci of the tail are not more than about half the length of the tail trunk.
4. The ventral sucker is situated near the posterior end of the body.
5. The oral sucker is provided at the anterior end with an invertible pouch or a protrusible snout.
6. Body and tail are covered with spines.
7. Body possesses no pigment or 'eye' spots.
8. Muscular pharynx absent.
9. Cæcum short and small.
10. Paired group of mucin glands around the ventral sucker.
11. Mucin ducts arising from these glands and entering the oral pouch.
12. Duct openings capped by hollow piercing spines.
13. A collection of germ cells present behind the acetabulum.
14. Excluding those in the body, a single pair of flame cells present in the tail.
15. Excretory canals open at the tip of the furcal rami.
16. Development in sporocysts.
17. No rediæ formation.
18. No encystment.

The above common characters are based upon our knowledge of the morphology of the cercariæ of the three human and one animal infecting schistosome : and looking to the close similarity in general characters of the various schistosome cercariæ so far known, it would not be unreasonable, excluding the avian forms, to expect similar characters to be present in other schistosome cercariæ also.

It may be noted that among others the cercariæ of *Sch. bovis*, *Sch. bomfordi* and *Sch. indicum* of the animal species, have yet to be discovered

and this table is intended to serve as a guide to their recognition when met with.

*Nomenclature.*—Although from a zoological standpoint the cercaria is merely a larva of the adult parasite and does not deserve a separate name; for the sake of convenience of description and reference, I would suggest the name of *Schistocercaria* to the group of *larvæ schistosomæ acuminatæ*, i.e. the identity with the adult schistosomes of which has been thoroughly established. The group will naturally be an expanding one including within its fold every new schistosome cercaria adult of which becomes definitely known. It will at the same time exclude those classed as schistosomal larvæ from their morphological characters alone, but whose identity with the adult has not been experimentally proved. Each individual of the above group may then be referred to by the addition of the specific name of the adult. Thus the cercaria of *Schistosomum japonicum* or the cercaria of *Schistosomum haematobium* may be referred to in short as *Schistocercaria japonicum* and *Schistocercaria haematobium* respectively.

#### SUMMARY AND DISCUSSION.

A detailed description is given of the morphological characters of the cercaria of *Schistosomum spindalis*, the identity of which was proved by raising the adult parasites from the larvæ and also by obtaining the larvæ from the adult. A comparison is made in the table of the characters of the cercariae of three human and one animal schistosome, and also a form described by Captain Sewell as *Cercaria indica*, type XXX, found in Calcutta. The inclusion of this form in the table has been necessitated by the fact that in his description of this cercaria, Captain Sewell asserts that it is almost identical with the cercaria of the Japanese schistosome and feels justified in raising an alarm as to 'the possible occurrence of *Sch. japonicum* Katsurada in India,' in the title of his paper. It will be interesting to see how far this view is justified or is warranted by the facts. As will be apparent from the table, the general morphological features of the four different schistosome cercariae are closely similar, and that such differences as exist, though important, are small. Again, it is difficult always to determine with certainty one of these small differences and other morphological details, e.g. in regard to the number of mucous glands to which Faust<sup>(\*)</sup> attaches diagnostic importance and states that the cercariae of *Sch. haematobium*, *Sch. muriei* and *Sch. japonicum* are easily distinguished on the basis of papillæ and

type of mucin glands and ducts, and their outlets; Cort<sup>(8)</sup> states that it is not easy to distinguish clearly the outlines of the cephalic (mucin) glands and to determine their exact number. In regard to the flame cells also the same difficulty of accurately determining their number is borne out by the varying descriptions of the excretory system of the cercaria of *Schistosomum japonicum* given by different observers: examples of these have already been given in the text. Thus not only are the differences minute, but even these are often too indefinite and uncertain. And it is likely that a wrong conclusion may be arrived at as to the identity of any cercaria by either ignoring the small differences or by inadequate morphological observations.

Comparing the new form described by Captain Sewell with the cercaria of *Sch. japonicum*, it will be found that in spite of its close resemblance in other respects it differs from the latter chiefly in measurements, in the character of the group of mucin gland cells, and in the nature of the host. Captain Sewell considers the first as negligible, regards the second as a physiological rather than a morphological difference, and makes no mention of the last. As regards measurements, it will be noted that variations in the cercariæ of the same species is very slight and that there is no evidence to show that it is greater in specimens from different parts of the world. A rough comparison of the several schistosome cercariæ depicted in Plate II, Fig. 6, will show that Captain Sewell's specimen resembles *Sch. mansoni* as much as it does *Sch. japonicum*. Regarding his assertion of the second difference being a physiological rather than a morphological one, it is worth noting that the same differentiation of the mucin gland cells into coarsely granular and finely granular or almost homogeneous cells has been observed, as has already been pointed out, in the cercariæ of *Sch. mansoni*, as well as in the present species (*Sch. spindalis*), and in this respect the species described by him would resemble these forms especially the latter more than the Japanese form.

As to the difference between Captain Sewell's specimen and the Japanese form in the nature of their intermediate host, it may be stated that although Cort<sup>(20)</sup> has shown that the forked-tailed cercariæ adapt themselves to new molluscan intermediate hosts, his examples of such adaption do not go beyond narrow limits of members of closely allied families—e.g., *Planorbis*, *Limnæa* and *Physa*. One would not be justified without some convincing proof, in assuming from this that a cercaria of *Sch. japonicum* which in Japan develops in an operculate mollusc

(*Hypodistomus nasopharyngealis*) of the Hydrobid family will adapt itself in India to non-operculate molluscs *Planorbis* and *Lamna* (in which Captain Sewell's specimen is stated to develop) belonging to an entirely different order, especially when there exist in this country operculate molluscs of a closely related family and capable of harbouring cercariae. It would thus appear that there are important morphological and other differences between the cercaria of *Sch. japonicum* and the species described by Captain Sewell. The Indian species cannot be taken as identical with the Japanese form in absence of a more convincing experimental proof. The suggestion made by Captain Sewell as to the possible occurrence of the disease in India, would, therefore, appear premature. Meanwhile the results of infection experiments which are stated to be in progress will be awaited with interest.

In conclusion, I desire to express my sincere gratitude to Lt. Col. Wm. Glen Liston, the Director, for the keen interest he has taken in the investigation, and for his constant help and encouragement, and valuable criticism.

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## 22 *Cercaria of Schistosomum Spindalis (Montgomery).*

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# EXPLANATION OF PLATES

## ABBREVIATIONS USED.

ANT.	=	anterior collecting tubule
AMG.	=	anterior group of mucin glands
C.	=	caecum
CA.	=	ciliated areas
DS.	=	duct spines.
EXP.	=	excretory bladder
EXP.	=	excretory pore
EXT.	=	excretory tubule in the tail
FC.	=	flame cell.
FR.	=	furcal rami.
GC.	=	germ cells.
HG.	=	heaf gland
I.	=	islet anastomosis.
M.	=	mouth
MD.	=	mucin ducts.
MX1	=	main excretory tubule
N.	=	nervous system
OE.	=	oesophagus
OS.	=	oral sucker.
PC.	=	paracytomatous cells.
PCT.	=	posterior collecting tubule.
PMG.	=	posterior group of mucin glands
ST.	=	stem of tail
CS.	=	central sucker.



## NOTES ON SOME FURCOCERCIOUS CERCARIAE FROM BOMBAY.

BY

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*From the Bombay Bacteriological Laboratory.*

(With Plates III—VI)

(Received for publication, October 20, 1920.)

### INTRODUCTION.

THE study of the Indian forms of furcocercous cercariae is of very recent date. During the present investigation which began about the end of 1916, one of the problems taken in hand was to ascertain whether any of the local molluscs was capable of acting as an intermediate host of human bilharzial disease of Egypt. It was necessary before undertaking these experiments to determine how far the indigenous molluscs were already naturally infected with trematode larvae. In the course of these investigations, large numbers of fresh water molluscs were examined and different types of furcocercous and other cercariae were discovered. The importance of the furcocercous group of cercariae lies in the fact that the larval forms of schistosomes or the bilharzial parasites of man, domestic animals, and birds belong to this group. A prominent feature of this group is the presence of a bifurcated tail. Although the general appearance of most of these furcocercariae is similar, minute differences in structure exist by which it is possible to differentiate one species from the other. A detailed study of the structure is, therefore, essential for the diagnosis of species as well as for purposes of comparison.

Faust<sup>(1)</sup> (quoted by Kemp and Gravelyn<sup>(2)</sup>) observes that "a very superficial description of the worm is a distinct barrier to the literature. The cercaria should be carefully studied in mounts, dead or not at all."

This paper contains a description of such of these furcocercariae as have recently been studied in detail. A lengthy description of each has, however, been omitted, as it was feared, it would entail much repetition owing to the close agreement in general features of most of these forms. It is, therefore, considered sufficient after having already given a detailed description of one of them—the cercaria of *Schistosomum spindalis*<sup>(3)</sup>—to briefly describe the main characters of others. Particular attention has been paid to the excretory system which is considered an important feature of these larvae and separate figures are given showing in detail the arrangement of this system in each type. No specific names but only provisional numbers are assigned to each cercaria.

*Cercaria bombayensis* No. 8.

(Plate III—Figs. 1, 2, 3, and 4.)

This is a small bifid-tailed cercaria with pigmented eye-spots. When viewed in water it is always seen with its body bent on one side and the tail turned upwards. This is the only forked-tailed cercaria I have met with which develops in rediae. The rediae are small and their anterior half is covered with spines. The muscular pharynx is prominent and the intestine is long and reaches the whole length of the structure. The infected liver has a dull greyish yellow colour. The sporocysts are small and have no club-shaped muscular ends.

The cercaria measures as under—

(Specimen killed by heat and measured without a coverslip)

Body	—	130	long	×	35 $\mu$	broad.
Tail trunk	—	240 $\mu$	..	×	24 $\mu$	..
Furcal rami	—	65 $\mu$	..			

The pigmented eye-spots are located 55  $\mu$  from the anterior end. They are composed of a central refractile lens surrounded by granular brownish black pigment. The body, tail, and the furci are all covered with sharp spines; these are especially marked over the conical anterior end. On the posterior half of the dorsal aspect of the body is attached a thin narrow membrane which when thrown into folds gives the appearance of spines. The alimentary system is simple, and, as in schistosome cercariae, consists of a mouth which opens a little behind the anterior end, a long oesophagus passing through the oral sucker, and a dilated caecum which is situated about 80  $\mu$  from the anterior end. There is no trace of any muscular pharynx. There is no well formed

# DESCRIPTION OF PLATE III.

FIG. 1. *Ceratomyxus homiogenus* No. 8 Ventral view.

- " 2. " " showing details of the structure of the body.
- " 3. " " showing details of the excretory system (semi-diagrammatic).
- " 4. Redia of the same

# DESCRIPTION OF PLATE III.

- Fig. 1. (General appearance, No. 8 Central view.)  
 2. showing details of the structure of the foot.  
 3. showing details of the excretory system  
 (semi-distended).  
 4. Redia of the same

# PLATE III.



Fig 2

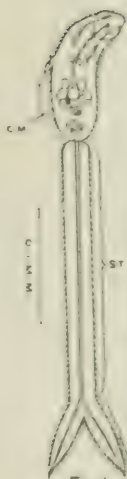


Fig. 1

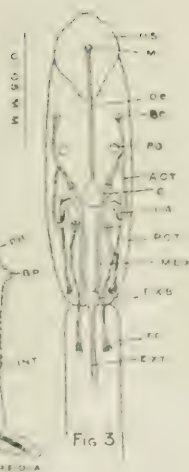


Fig 3



Fig 4



ventral sucker, but in its place is seen a mass of cells which appears as a rudimentary sucker. It plays no part in locomotion of the cercaria. Behind this rudimentary sucker is a cluster of small round cells representing the genital system. The oral sucker is situated at the anterior end and measures  $35\ \mu$  long, and is provided with a protrusible snout covered with papillae. It is smaller and less muscular than that in the cercaria of *Schistosomum splendens*. In the centre of it is situated the head gland with its granular contents.

Four pairs of main glands are located around the caecum and ducts from these pass forwards, and enter the oral sucker to open at the anterior end on either side of the opening of the head gland in the mediane. The opening of each duct is slightly dilated and is capped by a hollow pointing spine, similar to that noted in the cercaria of *Schistosomum splendens*.

The excretory system resembles that described for the cercaria of *S. splendens*. It possesses five pairs of flame cells including one pair in the tail. The four pairs in the body are located as under :

One behind the posterior margin of the oral sucker, the second on each side of the caecum, the third on each side of the rudimentary ventral sucker, and, the fourth at the posterior end of the body. The ciliated areas in the main excretory canals noted in the cercaria of *Schistosomum splendens* are also present in this species; no islet anastomosis, however, could be made out, and the openings of the branches at the end of the lateral ram appeared to be capped with a curved spine. Whether it has any connection with the excretory canal or not could not be ascertained. This species was found in the livers of *Labeo ananias* collected from puddles on the east of Mahim Railway Station in the suburbs of Bombay, in the middle of January, 1917. It was also, but very rarely, found in *Planorbis exustus* collected from Bandra (Bombay). Except for the absence of a well developed ventral sucker, this species being a pigmented aphyrogenous heterocercous cercaria, might be classed with the *Bilharziella* group of schistosomes. On account of the partially developed ventral sucker, however, its zoological position would be between a monostome and a distome. The rudimentary sucker indicates the probable line of evolution of these forms.

It is worth mentioning that attempts were made to the development of new adult parasites in the bodies of guinea pig and rabbit (see these records), but as was to be expected without success. On one occasion

the liver of a guinea-pig which was previously exposed to infection with this cercaria was found to contain spindle-shaped eggs resembling those of *Sch. spindalis*. Repetition of the experiment however gave uniformly negative results; and it was evident that in addition to the present species, the guinea-pig must unconsciously have been exposed to infection with the cercaria of *Sch. spindalis* also, which was probably present in the mollusc as a mixed infection and which had been overlooked.

*Cercaria bombayensis* No. 9.

(Plate IV, Figs. 1, 2, 3, and 4.)

This cercaria is much larger than the preceding one. It develops in long cylindrical sporocysts provided with club-shaped motile ends which enable them to sway from side to side. No rediæ have been noticed in this species. The infected liver has an orange brown colour. The cercaria was found in *Planorbis exustus* collected from a tank in Andheri near Bombay, in January, 1917. It has never been found in any other mollusc. There appears to be a seasonal variation in the number of snails found infected with this cercaria. Usually the mollusc contains only one type of cercaria but occasionally this species has been found to be associated with a xiphidio cercaria as a mixed infection, and on one occasion, as has been noted elsewhere<sup>(3)</sup>, the mollusc was found to harbour three different types of cercariæ—(1) cercaria of *Sch. spindalis*, (2) a xiphidio cercaria, (3) the present species. The larva has great powers of extension and contraction.

The measurements of specimens killed by heat are as follows :—

Body — 225  $\mu$  long by 52  $\mu$  broad.

Tail trunk — 325  $\mu$  „ „ 38  $\mu$  thick.

Furcal rami— 300  $\mu$  „ „

A prominent feature of this cercaria is the long sword-like furcal rami which are always held at right angles to the stem of the tail. The integument is covered with sharp spines which are thickly set over the anterior end. The body is provided with both an oral and a ventral sucker. The latter is situated at the junction of the middle with the posterior third of the body. It is circular in outline and has a diameter of 20  $\mu$ . Around the ventral sucker is a group of three pairs of mucin glands, one in front and two behind it. All the glands possess fine granular protoplasm and rounded nuclei. There is no differentiation

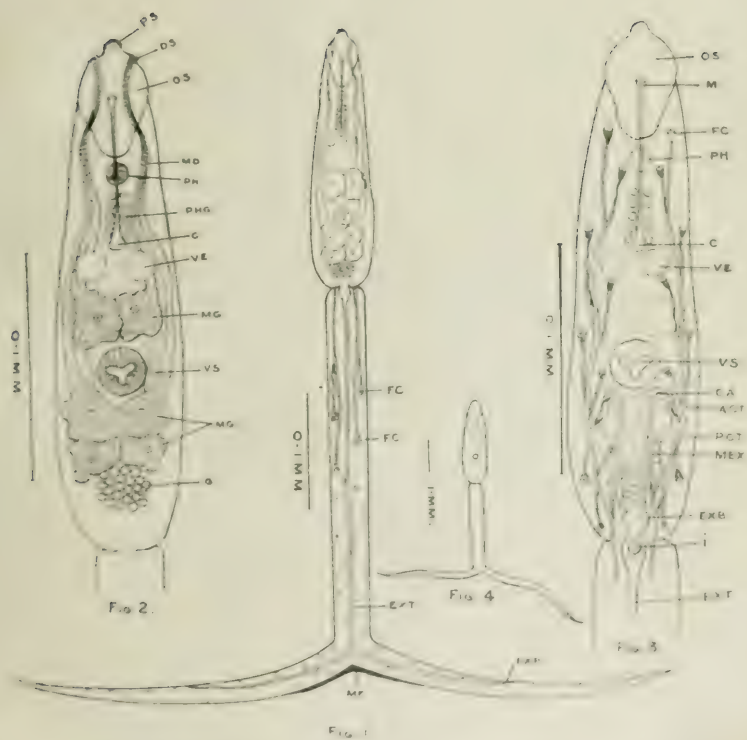
DESCRIPTION OF PLATE IV.

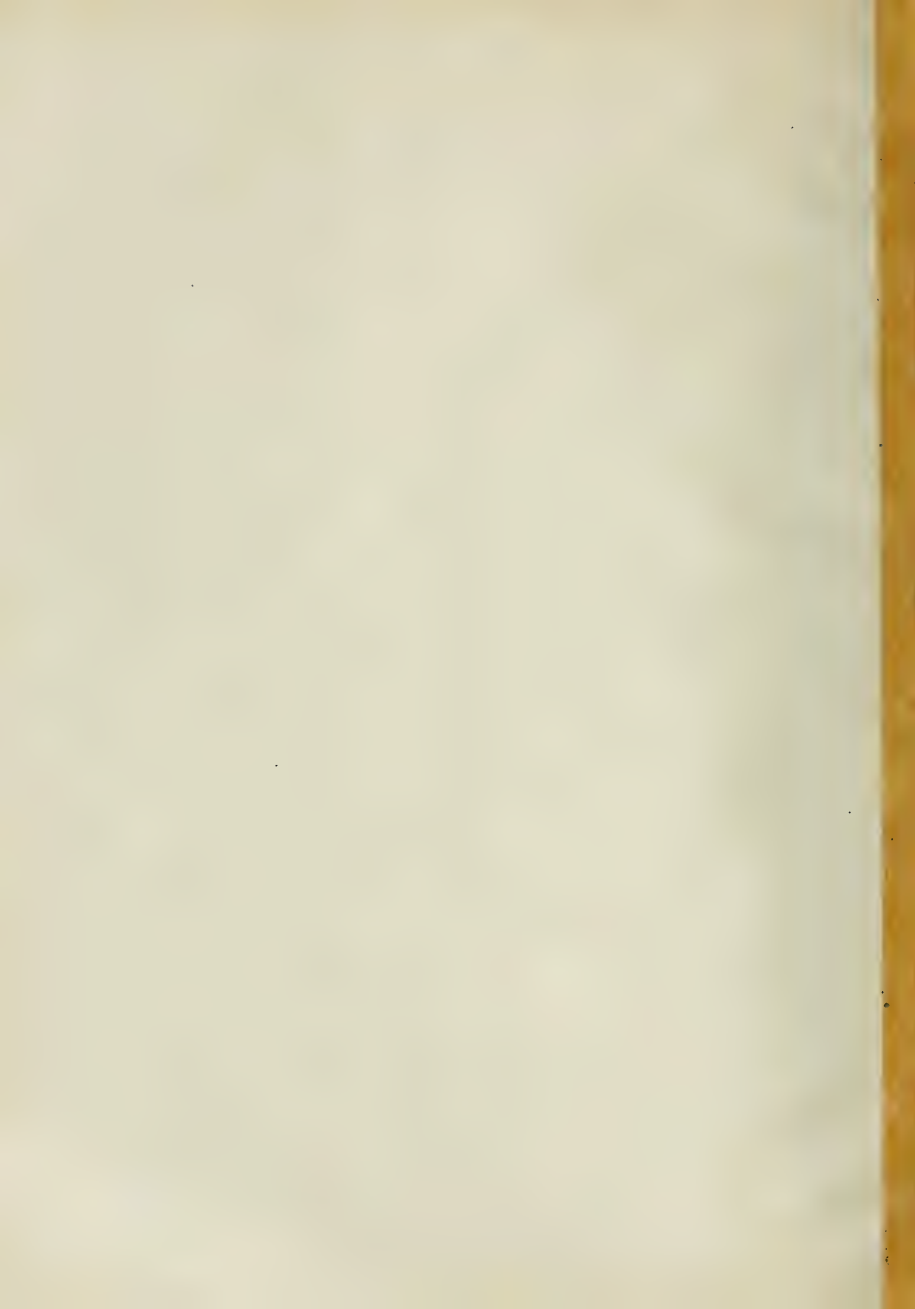
- Fig. 1. *Cercaria bombopense* No. 9 Ventral view.  
" 2. " " showing details of the structure of the body.  
" 3. " " showing details of the excretory system  
(semi-diagrammatic).  
" 4. " " as seen under a low power microscope.

DESCRIPTION OF PLATE IV

- Fig. 1. (A) *Anterior view of the head of the fly*  
 " 2. *showing details of the structure of the body*  
 " 3. *showing details of the excretory system*  
 " 4. *(semi-diatomaceous)*  
 " 5. *as seen under a low power microscope*

# PLATE IV.





between coarsely granular and finely granular cells in this as in the cercaria of *Sch. spindulus*. The granules are neutrophile in their staining reaction. The glands are brought out prominently by the method of intravital staining. Ducts from these glands pass forwards and enter the oral sucker and eventually open externally at the anterior end, their tips being capped with hollow spines. There are only three pairs of ducts and three pairs of spines capping them. Just in front of the anterior pair of mucous glands is seen a pair of cells having a pale yellowish colour. These cells probably represent the vestigial eye spots similar to those described by Faust<sup>(4)</sup> for *Cercaria guineensis*. No lens could be detected nor could any granules be seen in it. The oral sucker is a strong muscular organ measuring  $45\ \mu$  by  $30\ \mu$ . Its general features are similar to those present in the schistosome cercariae. It is provided with a protrusible snout which when pushed out is seen to be covered with numerous large sharp spines.

The mouth is located a little behind the anterior end and the oesophagus passes through the oral sucker and opens in a bulbous caecum situated a little in front of the yellowish eye spots. Just below the posterior margin of the oral sucker, the oesophagus is surrounded by a muscular pharynx, and beyond this point, it, as well as the caecum, are lined by a row of rounded gland like structures. (Plate IV, Fig. 2 and 3.)

The arrangement of the excretory system resembles that of the schistosome larva in many respects. The muscular bladder at the posterior end of the body gives rise to two large trunks anteriorly, each with two fusiform dilatations containing ciliated areas. It is continued posteriorly into the tail as a single trunk which bifurcates to enter the furcal vent and opens in the middle of the furca and not at the tip as in the *Sch. spindulus* larva. (See Plate IV Fig. 1.) This sort of an arrangement resembles that seen in *Cercaria emarginata* and *C. douglasi*. [See Cort (5).] The characteristic islet anastomosis is also present.

There are two pairs of flame cells in the tail, which are situated about its middle, and the body contains ten pairs of flame cells: the largest number yet observed in a furcocercous cercaria.

These are located as below :

Beginning from the most anterior,

1st, at the posterior margin of the oral sucker.

2nd, on each side of the pharynx,

3rd, on each side of the cæcum.

4th at the posterior margin of the eye-spots.

5th, at the posterior margin of the anterior pair of mucin glands.

6th, at the anterior margin of the middle pair of mucin glands.

7th, at the posterior margin of the middle pair of mucin glands.

8th, at the posterior margin of the posterior pair of mucin glands.

9th, at the level of the middle of the main excretory trunk.

10th, on either side of the excretory bladder.

The whole arrangement of the excretory system is shown in Plate IV, Figs. 1 and 3.

The genital system is represented by a mass of germ cells behind the acetabulum.

The movement of this cercaria differs from that of the cercaria of *Schistosomum spindalis*. When resting in water it is seen with its body doubled up and the prongs wide apart. It swims with great rapidity in all directions in a winding sort of a manner thus differing from the cercaria of *Sch. spindalis* which usually swims in a perpendicularly upward direction. The motion again is not continuous, but intermittent, and both the start and the stop are quite sudden, and it is often possible even with the aid of a hand lens alone to tell one species from the other by its characteristic movement. On a substratum, it merely extends and retracts its body, but is unable to crawl, and there is no looping movement characteristic of the schistosome cercaria. Both the body and the tail are capable of rapid vibration and the furci are moved by a strong layer of muscle fibres situated in the notch of the bifurcation.

This larva does not encyst.

This species has some resemblance to *Cercaria gladii* found by Cawston and described by Faust<sup>(6)</sup>. The main points of resemblance are the long sword-like furcal rami, the three pairs of mucin glands, and sporocyst with a muscular anterior end. Measurements are also somewhat similar. No description of the excretory system which forms an important feature of this cercaria has, however been given of *Cercaria gladii*.

The molluscan host of *Cercaria gladii* is again different being found in *Isidora schakoi* in Transvaal, while the present species is found in *Planorbis exustus*. The presence of a muscular pharynx in the present species at once puts it out of the group of schistosomid larvæ. Bahr and Fairley<sup>(7)</sup> have also described a somewhat similar cercaria from *Bullinus dybowski*, but its measurements are much smaller.



# PLATE V.

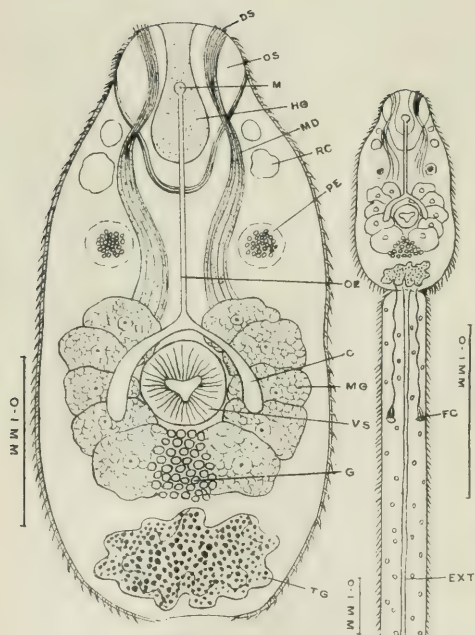


FIG. 2.



FIG. 4.

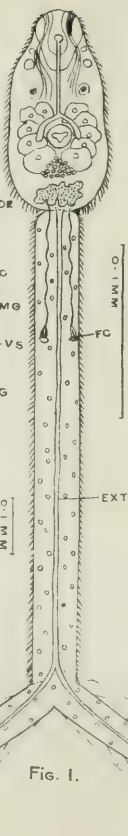


FIG. 1.

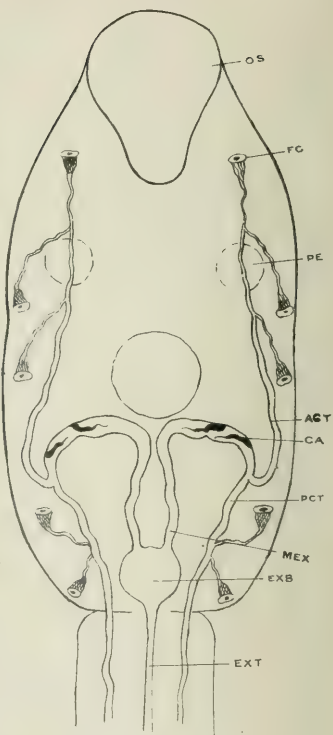


FIG. 3.



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DESCRIPTION OF PLATE V

Fig. 1	<i>Uca</i>	showing details of the structure of the body.
Fig. 2	"	showing details of the excretory system.
Fig. 3	"	(semi-diagrammatic)
Fig. 4	"	as seen while swimming in water

Fig. 1 *Uca* (body) No. 13 ventral view

DESCRIPTION OF PLATE V

Fig. 1. *Cercaria bombayensis* No. 13 ventral view.

" 2. " " showing details of the structure of the body.

" 3. " " showing details of the excretory system (semi-diagrammatic).

" 4. " " as seen while swimming in water.

*Cercaria bombayensis* No. 14

(Plate V, Figs. 1, 2, 3, and 4)

This is a pigmented type of furcocercous cercaria, developing in small cylindrical sporocysts not provided with any muscular club-shaped end, and devoid of movement. The infected liver has a light yellowish-brown colour. This species was found in *Plumulus vigatus* in a tank near Andheri, near Bombay, in August, 1917.

This cercaria when swimming in water in a test tube appears very similar to the preceding one (*C. bombayensis* No. 9), the body being doubled upon itself (See Plate V, Fig. 4); but in this species the tail also remains bent. In water it gradually sinks by its own weight, when suddenly it starts off swimming in all directions.

Its body measures  $350\ \mu$  long by  $185\ \mu$  broad.

The tail trunk is  $850\ \mu$  long,  $80\ \mu$  thick.

The furca measure  $350\ \mu$  long.\*

The whole larva measures more than 1.5 m.m. in length. Both the body and the tail are covered with spines, and the furcal rami have a thin membrane attached to them. The ventral sucker is a very prominent structure and is situated just behind the middle of the body, and forms a marked projection when the cercaria lies as it often does, on its side, the projection often reaching  $35\ \mu$  beyond the general surface. Surrounding the ventral sucker are several mucin glands with finely granular protoplasmic contents. It is rather difficult to determine their exact number, but as far as one could make out there appeared to be five pairs of glands. Ducts from these enter the oral pouch and open externally, their tips being capped by hollow piercing spines as in the cercaria of *Sch. spindalis*. Behind the ventral sucker is a cluster of cells, the rudimentary genital system, and behind this is a large irregular cell with coarse granules which takes a deep stain when coloured by the intravital method. It is difficult to say what function this large gland-like structure performs. No duct arising from it could be made out. (See Plate V, Fig. 1, vc.)

The oral sucker measures  $100\ \mu$  in length by  $50\ \mu$  at its broadest part and is divided as in schistosome cercariae into an anterior and a posterior portion. In the centre of it is contained the head gland and on either side of it run the ducts of the mucin glands. Outside the oral sucker

\* These measurements were taken from living specimens. At killing by fast freezing considerable wrinkling, satisfactory measurements could not be taken by this method.

between it and the body wall on either side are situated a couple of large refractile cells. The significance of these is not known. The mouth is situated a little behind the anterior end, and the narrow œsophagus passing through the posterior portion of the oral sucker is continued backwards up to the anterior margin of the ventral sucker where it opens into a slightly dilated bifurcated cæcum, the limbs of which curve round the sides of the ventral sucker. The œsophagus is not provided with any muscular pharynx. Between the ventral sucker and the posterior margin of the oral sucker are situated two large pigmented eye-spots. The pigment is in the form of dark granules and in the centre of this mass is located a refractile lens.

The general arrangement of the excretory system resembles that of the schistosome cercariæ. The two branches of the excretory trunk in the tail open at the tip of the furcal rami. There are five pairs of flame cells in the body and one in the tail, the latter is situated further towards the distal end than is the case in the cercaria of *Sch. spindalis*. The flame cells in this species are also proportionately larger.

*Cercaria bombayensis* No. 19.

(Plate VI, Figs. 1, 2, and 3.)

This cercaria was found on a single occasion on 12th April, 1920, in a tank at Andheri in the suburbs of Bombay, in the liver of *Limna acuminata*. It is a furcocercaria with pigmented eye-spots and belongs probably to the 'Bilharziella' group of cercariæ.

The measurements of specimens killed by heat are as follows:—

Body—380  $\mu$  long by 80  $\mu$  broad.

Tail trunk—435  $\mu$  long by 58  $\mu$  thick.

Furci—290  $\mu$  long by 28  $\mu$  broad at the root.

In living specimens the body when fully extended measured 450  $\mu$  in length while it could contract down to only 250  $\mu$ . The tail and the furci also showed variation but not to the extent shown by the body; the length of the tail varied between 350 and 450  $\mu$  while that of the furci between 220 and 300  $\mu$ .

The body and the tail are covered with spines. The ventral sucker is situated about the posterior third of the body. It is a strong muscular structure and forms a marked projection when the animal is lying on its side. It is about 20  $\mu$  in diameter and 25  $\mu$  in depth. It is surrounded by a group of four pairs of mucin glands from which ducts arise to pass forwards and enter the oral sucker, and ultimately open

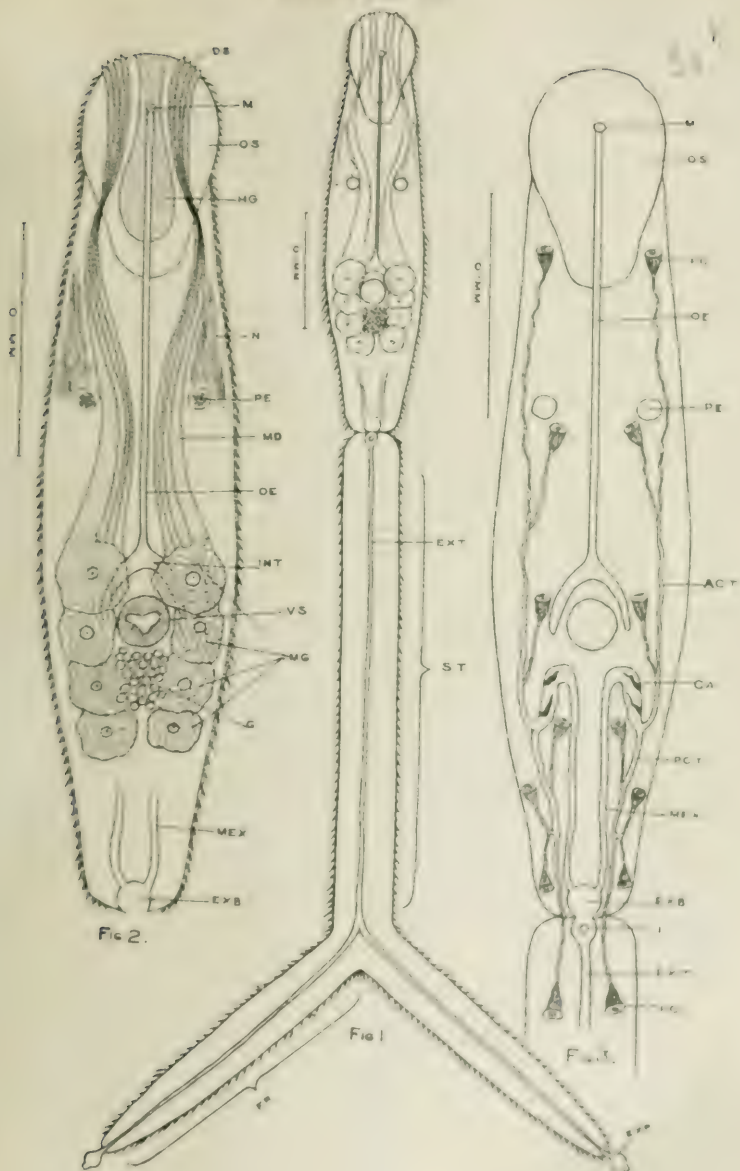
DESCRIPTION OF PLATE VI.

- Fig. 1. *Cocoonia bambusa* No. 19, the whole animal, ventral view.  
.. 2. " " " showing details of the structure of the body.  
.. 3. " " " showing details of the excretory system  
(semi-diagrammatic).

# DESCRIPTION OF PLATE VI.

Fig. 1. *Cercaria bombayensis* No. 19, the whole animal, ventral view.  
 " 2. " showing details of the structure of the body.  
 " 3. " showing details of the excretory system  
 (semi-diagrammatic).

# PLATE VI.





externally at the anterior end on each side of the middle line, their expanded ends being capped as in the cercaria of *Sch. spindulifer*, by hollow pointing spines. The oral sucker is large measuring  $105\ \mu$  to  $60\ \mu$  and is divided into an anterior and a posterior portion, the latter being strongly muscular. This species also is provided with a protrusible snout. The centre of the oral sucker is occupied by the food gland and on each side of it run the mucin ducts. Between the oral and the ventral suckers are situated two pigmented eye-spots, each composed of a cell containing a central lens surrounded by numerous small brownish-black granules.

Movement of the cercaria is characteristic of the furcocercariae. It hangs in water with the head end downwards and swims backwards, but when it crawls on a substratum, the movement is forwards. Often during slow movement a constriction is produced about the middle of the body and the movement of the part in front gives an impression as if the animal were trying to escape from a narrow mouthed pouch.

Pharyngeal system is similar to that of the preceding species *C. benderupensis* No. 13. The mouth is situated a little behind the anterior end. The long oesophagus without any muscular pharynx, and the bifurcated caecum reaching on each side of the ventral sucker can be clearly made out. Behind the ventral sucker and between the mucin glands on either side, is a cluster of cells—the rudimentary genital system.

The nervous system is represented by two large lobes just in front of the pigmented eye-spots. The general arrangement of the excretory system is typical of the schistosome group of cercariae, and need not be described in detail, a few main characters may alone be noted. The body possesses six pairs of flame cells in addition to one in the tail. The excretory pores open at the tip of the lateral margin, the openings being fluted into peculiar bulbous expansions projecting beyond the tip. The islet anastomosis in the bladder and the two ciliated areas in the main excretory trunks are also present. The whole arrangement is shown in Plate VI, Figs. 1 and 3.

The infected liver has a dark brownish-yellow colour. These cercariae develop in elongate sporocysts having no club-shaped caudal end. There is no stage of redia formation, each sporocyst containing about 5–8 cercariae in various stages of development. These sporocysts are not firmly attached to the organ in which they develop so that (unlike the

sporocysts could easily be teased out for examination. They measure about 1.5 m.m. long by .15 m.m. in thickness and their dark appearance is often due to numerous dark granules embodied in the walls. The cercaria does not undergo encystment.

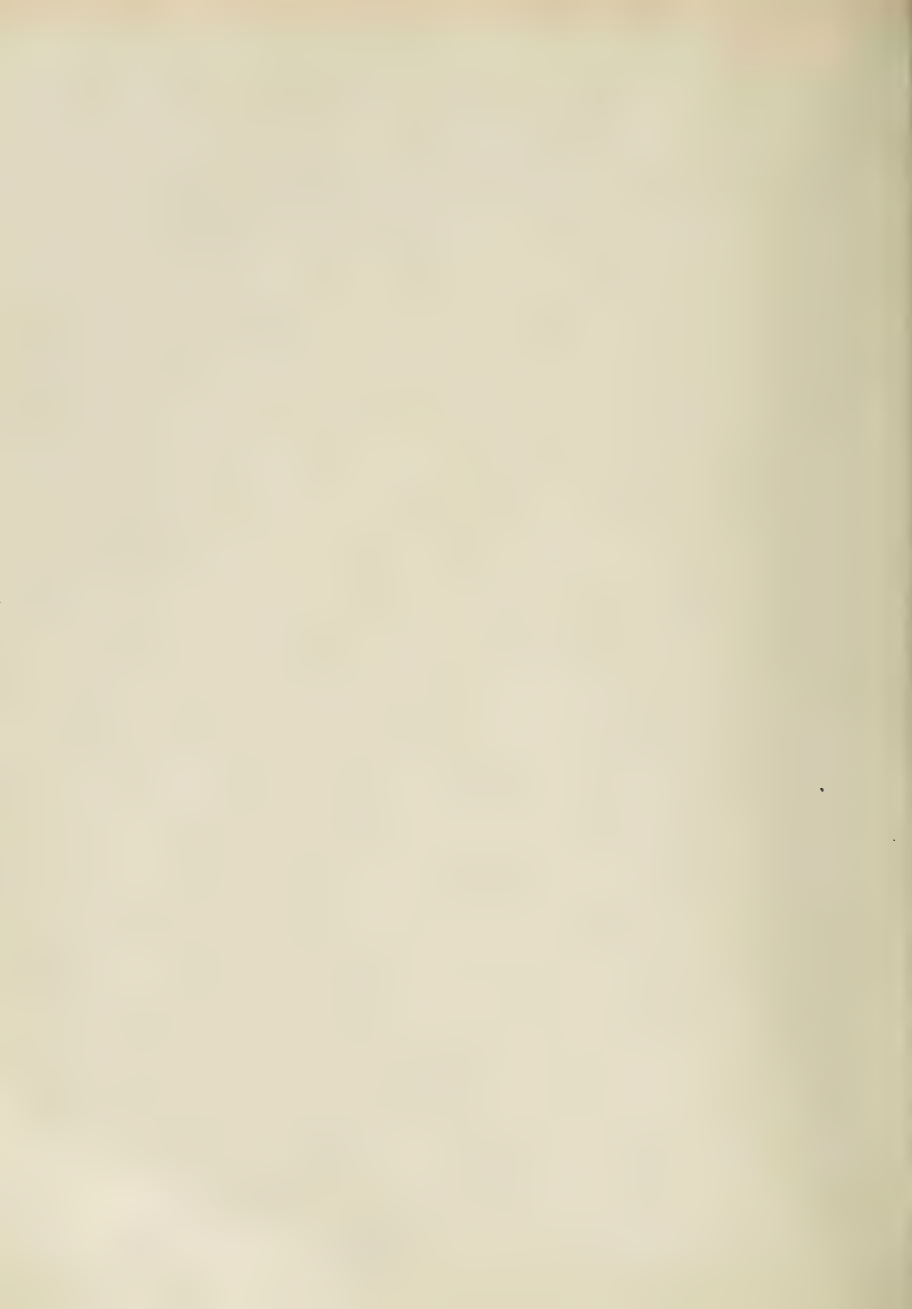
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# EXPLANATION OF PLATES

## ABBREVIATIONS USED

act	= anterior collecting tubule
ap	= anal pore
cl	= cilium
ca	= ciliated areas
cm	= ciliated membrane
ds	= duct, ducts
exg	= excretory gland
exp	= excretory pore
ext	= excretory tubule in the tail
fc	= flame cell
fk	= filarium
gc	= germ cells
gl	= gland
j	= jet, anastomosis
int	= intestine
lt	= lip, lips
md	= median ducts
mg	= mucous glands
met	= main excretory tubule
mf	= muscle fibres
n	= nervous system
he	= nephridia
os	= oral sucker
pc	= posterior collecting tubule
pe	= pigmented eye spots
ph	= pharyngeal pharynx
phg	= pharyngeal glands
ps	= protrusible snout
sc	= setaceous cell
rvs	= rudimentary ventral sucker
st	= stem of tail
tg	= testicular gland
ve	= ventral eye spot
vs	= ventral sucker



THE CORRELATION BETWEEN THE CHEMICAL  
COMPOSITION OF ANTHELMINTICS AND  
THEIR THERAPEUTIC VALUES IN  
CONNECTION WITH THE  
HOOKWORM INQUIRY  
IN THE MADRAS  
PRESIDENCY.

BY

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(Received for publication, November 26, 1920.)

X. BETANAPHTHOL.

HISTORY.

A. THE treatment of hookworm infection with betanaphthol was introduced some twenty years ago and was first adopted in Assam Estates, where it seems to have in a very short time superseded all the other treatments then in vogue.

Early in 1904, Bentley<sup>1</sup> published the results of his investigation into the comparative values of several anthelmintics used in the treatment of ankylostomiasis. The four drugs which appeared to him most useful were, in the order given, betanaphthol, thymol, pulegia urens, and extractum filicis. Of the two plants his preference

went to betanaphthol as being pleasanter to take, used in smaller doses, equally or more efficient, useful in pernicious anemia, not so irritating to the alimentary canal, and not followed by unpleasant giddiness.

Castellvi (1908)<sup>4</sup> successfully treated his cases from the lead and silver mines of Linares, in Spain, with betanaphthol, and regretted that the superior efficiency and harmlessness of that drug had not yet been properly recognised. With all deference to Castellvi it must, however, be admitted that betanaphthol had, by this time, become for many the only drug worth consideration in the treatment of ankylostomiasis. Its use was favoured by Colbert<sup>5</sup> in New Mexico, Burton Nicol<sup>8</sup> in Natal, Burkitt<sup>7</sup> in India, and Drummond<sup>6</sup> in Ceylon. It is moreover evident from the report and proceedings of a Committee<sup>11</sup> appointed in Ceylon to consider and advise as to what measures should be taken to prevent the spread of hookworm infection, that most of the medical officers in the island had adopted methods of treatment with betanaphthol which were but modifications of the method followed in Assam. By the end of that same year 1908, the Indian Immigration Board of Natal had decided in favour of the drug and were to remain faithful to its use in spite of adverse criticism from the Commission in Porto Rico.<sup>2</sup> Yet the indictment was of an alarming nature:—betanaphthol was said to be a powerful kidney irritant, to keep very badly, to be variable in quality, to be unsuitable in large doses for out-of-door or dispensary practices.<sup>12</sup>

From that time onwards, the efficacy of the drug was repeatedly asserted, Thomas<sup>10</sup> being the only one to have complained of its not having acted well in his hands. In 1911, betanaphthol had become, so to speak, a household remedy for hookworms in the Federated Malay States.<sup>26</sup> In 1912, Cockin<sup>16</sup> pointed to the undoubted superiority of betanaphthol, not only on account of its rapid and complete expulsion of the parasites, but also because of its cheapness and the freedom from danger in its administration. Bryson (1913)<sup>18</sup> gave preference to the drug as being most satisfactory for routine purposes.

With the advent of oil of chenopodium, literature became practically silent about betanaphthol and its field history. All we can say is that the drug has not been altogether discarded and that it is still being used in some of the infected areas.

B. The smallest dosage recorded is that adopted by D'Almeida (1913)<sup>19</sup> who gave 33 centigrammes of betanaphthol combined in a

tabloid with 7 centigrammes of phenolphthalein as a purgative, the preliminary purge being omitted.

Perry (1915)<sup>27</sup> recommends betanaphthol as a substitute for thymol and, following Ferguson's method of administering thymol in British Guiana, advises a daily cachet of 10 grains of the drug every evening except on Saturdays, the coolie doing his work as usual. In the belief that the ingestion of alcohol after the treatment might lead to poisoning of a serious character, Saturday was declared *dry non*, as no amount of argumentation could keep the average coolie away from the toddy shop on that particular night.

Perry had previously (1910)<sup>11</sup> advised betanaphthol in 30 grains dosage. Coolies were separated into two groups:—(a) the new arrivals; (b) the residents of longer standing. The new arrivals received 15 grains (three tabloids) at 6 A.M. on an empty stomach, and a second dose of 15 grains the same day one hour before the evening meal. Half ounce of Epsom salts dissolved in a little water was given on the following morning. Residents of longer standing were treated with a dose of 30 grains (six tabloids) at 6 A.M. on an empty stomach and half ounce of Epsom salts the following morning; a second dose of 30 grains at 6 A.M. on the seventh day after the first dose, followed by half ounce of Epsom salts on the next morning. In carrying out this treatment attack and oil were to be entirely prohibited.

Castellvi (1908)<sup>4</sup> gave two grammes divided in two doses with an hour's interval between them—eight hours after a saline purge, followed two hours later by rhubarb and senna. The treatment was given every four or five days.

Ashford (1913)<sup>17</sup> treated thirty hospital cases with two grammes of betanaphthol. In the evening a dose of either magnesium or sodium sulphate was prescribed, care being taken not to give an exhaustive purge. The next day the patient was kept in bed without food till 1 P.M., 1 gramme betanaphthol, in capsule, was taken at 8 and 10 A.M., and at 12 noon another purge of salts. In the afternoon a light diet of milk was permitted.

Clayton Lane<sup>28</sup> in his Darjeeling Campaign (1916) used 30 grains betanaphthol given in three parts at hourly intervals, preceded with a dose of sulphate of magnesia, if possible, and immediately followed by a dose of this salt.

Darling, Barber and Hacker<sup>32</sup> report having treated 100 cases with betanaphthol, 20 grains, in capsules, two doses with two hour interval,

Schuffner and Vervoort (1913),<sup>21</sup> Day and Ferguson (1914)<sup>23</sup> administered 3 grammes in three hourly 1 gramme doses. And Greisert (1913)<sup>20</sup> summarising the reports of surgeons in East Africa, who had each treated some hundreds of cases with four different drugs—betanaphthol, thymol, eucalyptus, and male fern—concludes that betanaphthol is best in 15 grains doses given every day for three days; no supervision of the patient is necessary.

The Assam Method,<sup>6</sup> which seems to have been adopted later in the Federated Malay States, required the use of a 60 grains dosage divided in three doses of 20 grains:—

First day	..	4 P.M., a feed of rice.
		8 P.M., one ounce castor oil.
Second day	..	6 A.M., 20 grains betanaphthol.
		8 A.M., sago and salt.
		10 A.M., 20 grains betanaphthol.
		12 Noon, sago and salt.
		2 P.M., 20 grains betanaphthol.
		6 P.M., a feed of rice.
		10 P.M., castor oil.

The same programme of treatment was also followed in Ceylon, but the dosage was enhanced to 90 grains on the Sheen and South Pundaluoya Estates and reached 120 grains at Dunsinane.<sup>11</sup>

Burton Nicol<sup>15</sup> used either 60 or 90 grains dosages. It is this latter dosage, administered in 30 grains doses every two hours for six hours, which came to be known as the Natal Method.

Whyte (1916)<sup>30</sup> gave two doses of 40 grains each with pre- and after-purge.

Burkitt (1909)<sup>7</sup> kept the patient's bowels open by pre-purges and used 90 grains betanaphthol in three 30 grains doses every two hours, and castor oil two hours after the last dose. The patient was starved from the previous evening to 3 P.M. on the next day.

Bayma and Alves (1918)<sup>31</sup> administer a preliminary saline purge and, on the next day, 6 grammes of betanaphthol in divided doses: 1 gramme every fifteen minutes until 6 grammes are taken. A final saline purge is given two hours after the last capsule of betanaphthol. The patients are treated in a hospital under careful supervision.

Gonzaga and Lima (1918)<sup>33</sup> omit the preliminary purge and 6 grammes of betanaphthol are given, all at one time, early in the morning, and the dose is repeated for three successive days. Throughout

treatment the patient receives only a light diet. Two hours after the last dose of betanaphthol a saline purge is administered.

C. The Porto Rico Anæmia Commission<sup>2</sup> were the first to point to the toxic action of betanaphthol on the kidneys, and they found that 83.3 per cent of their cases had albuminuria.<sup>12</sup> Landeman (1910),<sup>9</sup> Schalling (1911),<sup>14</sup> Ashford (1913),<sup>17</sup> Clayton Lane (1916),<sup>14</sup> also called attention to the poisonous effect of the drug upon the kidney. Cockin (1912)<sup>16</sup> and Howard (1918)<sup>34</sup> have recorded cases of hemoglobinuria.

Friedenwald and Leitz (1911)<sup>13</sup> found that in dyspeptics and in hyperchlorhydric cases betanaphthol slightly interferes both with the motor and secretory functions of the stomach. According to Van der Hoeve (1913),<sup>22</sup> the drug, when used in large quantities, may cause hypæsemia and even degenerative changes in the retina. Smilie (1920)<sup>36</sup> notes that large doses, 18 grammes, may produce very severe toxic symptoms associated with a destruction of red blood cells.

Geatling (1915)<sup>29</sup> cites the case of a Javanese male, just recovered from pneumonia, who was treated with 90 grains of betanaphthol in three 30 grains doses. Three days after the treatment vomiting, headache, jaundice, and dark red urine with albumin; temperature 101.8; no malaria. The patient was treated as for blackwater fever with Sternberg's Mixture, and all symptoms cleared. A second and a third treatment with betanaphthol caused no poisoning symptoms.

A fatal poisoning case due to betanaphthol is recorded by Orme (1915).<sup>25</sup> A Chinaman was given 5 grains of calomel one evening and half ounce of Epsom salts two days after. The next day, on August 29th, three 30 grains doses of betanaphthol were taken and were followed by Epsom salts. This treatment was repeated on August 30th. On August 31st, the patient started vomiting soon after the administration of the first dose. There was temperature; vomiting continued next day; dark urine, and slight jaundice. September 1st, the patient was much collapsed and on 2nd he died. Before treatment the urine was acid, had specific gravity 1010, and contained a small amount of albumin.

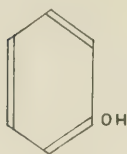
#### COMPOSITION AND PREPARATION

Betanaphthol, melting at 122° and boiling at 280°, crystalline or colourless or pale buff coloured shining lamellæ and dark purple melt

with a faint phenol-like odour. It is sparingly soluble in cold water—1000 parts—, fairly soluble in boiling water—75 parts—, soluble in 90 per cent alcohol 2 parts—, very soluble in ether, chloroform, glycerin, oils and fats, soaps and alkaline solutions. It is a drug of uniform quality and one that keeps well almost indefinitely.



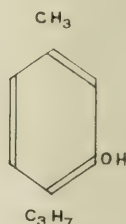
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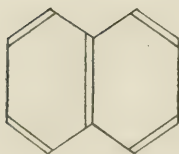
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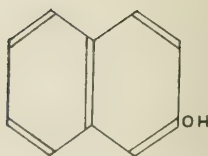
P-CYME



THYMOL



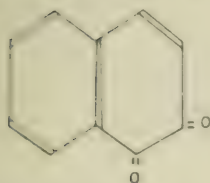
NAPHTHALENE



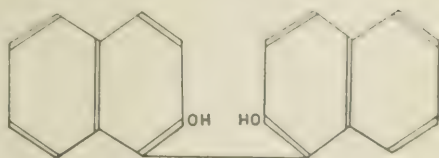
BETANAPHTHOL

Betanaphthol, or betamonohydroxy-naphthalene, bears to naphthalene the same relation that phenol does to benzene, and thymol to paracymene.

Betanaphthol is not directly oxidised to naphthoquinone, but it gives with ferric chloride a greenish-white dinaphthol, which may also be obtained by reduction of naphthoquinone.



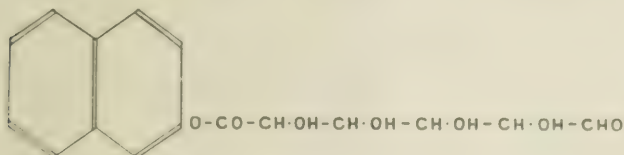
BETANAPHTHOQUINONE



DINAPHTHOL

Mono- and disulphonic acids result from the reaction of betanaphthol with sulphuric acid. The latter combine readily with diazocompounds forming red colouring substances.

Betanaphthol and glycuronic acid condense with elimination of water.



BETANAPHTHOLGLYCURONATE

Betanaphthol is a powerful antiseptic and germicide. Much less toxic than phenol and cresol it may be taken internally in fairly large doses without any toxic manifestations. When taken in too large quantities it acts as a caustic irritant of the mucous membrane and may cause gastric disturbance.

#### ABSORPTION AND ELIMINATION.

*Previous investigations.* Ashford and Igarcia-deg (1906) stated that the diazo reaction was invariably positive after the administration of betanaphthol in 1 or 2 grammes dose. Its presence was demonstrable three to four hours after the dose, and its effect lasted five or six hours.

The next day it was always absent. Apparently betanaphthol did not affect the colour of the urine. The reaction was practically always acid, rarely alkaline or neutral as Calmette found in his cases.

Schultz (1911)<sup>14</sup> states that betanaphthol is absorbed partially in the intestine and is expelled as betanaphtholglycuronic acid and betanaphthoquinone, and possibly in other slightly modified forms.

*Procedure adopted.* Healthy male convicts from the Trichinopoly Central Jail were selected for the purpose of our investigation. They were given a light diet at 5 P.M. followed by a magnesium sulphate purge at 8 P.M. The next morning they were asked to pass urine and were then treated with betanaphthol. Magnesium sulphate was given two hours after the administration of the last dose.

The urine discharged after the treatment was collected and clinically examined for albumin. Note was also taken of the colour, volume, density and acidity.

The species, number, and the condition of the hookworms expelled after the betanaphthol test-treatment were recorded, and the patient's total hookworm content was determined by repeating the thymol standard treatment until cure followed.

Variations in the dosage of the anthelmintic and in the mode of treatment were tried.

*Experiments.*—1. The different samples of betanaphthol we have been using were all found to be chemically pure. One of the samples had been bought 36 years ago and stored ever since in the laboratory where the temperature varies from 26° to 36°C.

2. Urines passed after treatment with betanaphthol did not give the standard reactions characteristic of that substance.

3. All attempts at separating betanaphthol in the free state failed.

4. The following colour-reaction, which never obtained with normal urines—50 cases examined—, was invariably positive after treatment with betanaphthol—81 cases:—To 2 c.c. of urine add an equal volume of basic lead acetate, and filter. To 1 c.c. of the filtrate add ten drops concentrated sulphuric acid, and heat to boiling. Allow the lead sulphate precipitate to settle down, and treat with one drop Milton's Reagent. With naphtholated urines a pink colouration is produced.

So far we have entirely depended on this reaction for the study of the absorption and elimination of betanaphthol.

RESULTS:—*Time taken for complete elimination of betanaphthol*:—50 grains betanaphthol were administered in two portions to 84 cases.

(a) In 68 cases the urine was collected for every period of 24 hours from the time of the administration of the first dose. The colour reaction we have described was invariably positive.

(b) In 16 cases the second day urine from 24 to 48 hours after the administration of the first dose was examined for betanaphthol and the colour-reaction was invariably negative.

(c) In 16 other cases the urine was collected on the first day at regular intervals of four hours, for a period of twelve hours. The night urine formed a separate fourth lot. It was thus found that betanaphthol is eliminated during the twelve hours which follow the treatment.

TABLE I.

*Betanaphthol elimination at different periods (by hours) after treatment with 50 grains in two doses.*

Case number	TIME OF URINE COLLECTION AFTER BETANAPHTHOL TREATMENT					
	0-24 hours	0-4 hours	4-8 hours	8-12 hours	12-24 hours	24-48 hours
1-84						
69, 76, 77		+	++	+	0	
70			++	+	0	
71, 84				+	0	
72		++	++	+	0	
73		+	++	+	0	
74		0	++	+	0	
75, 82			++	+	0	
79		++	++	+	0	
80		+++	+	+	0	
81		++	++	+	0	
84		+++		+	0	
85-88						

As seen from the table :

(i) Betanaphthol is absorbed in the system and eliminated by the kidneys.

(ii) Absorption is relatively slow.

(iii) Elimination is rapid, very rarely extends beyond the twelve hours, and never beyond the twenty-four hours which follow the treatment.

These results confirm the findings of Ashford and Igaravidez based on the diazo-reaction.

2. *The absorption of betanaphthol and its toxicity.*—In the course of our investigation betanaphthol has proved throughout a very safe drug. Very few toxic symptoms were noted and they were of a mild nature.

3. *Naphtholated urine.*—As a rule, both volume and density were normal, and the reaction acid. The presence of glycuronic derivatives did not prevent decomposition. The odour was in no way characteristic. The colour varied from pale straw yellow to light brown in the 24 hours collection, but in the smaller lots collected every four hours during the twelve hours which followed the administration of the first portion of the drug, the second and third samples were invariably dark brown or mahogany red, in striking contrast with the usually colourless first sample and the yellow urine of the night. The colour was due to betanaphthoquinone.

Indican was generally absent. 340 cases were carefully examined for albumin and no albuminuria was found to have occurred.

#### THE RATIONALE OF BETANAPHTHOL TREATMENT WITH REFERENCE TO ITS CONSTITUTION, ABSORPTION, AND ELIMINATION.

The points of similarity between betanaphthol and thymol are very striking :

(a) both are monohydroxy derivatives of hydrocarbons, the hydroxy radical being attached to a nuclear carbon atom ; and the nucleus being in both cases a true benzenoid structure ;

(b) they are both eliminated through the kidneys in conjugation with glycuronic acid ; but while thymol condenses without the

elimination of water, condensation in the case of betanaphthol takes place with elimination of water :

(c) the elimination is rapid with the two drugs and slightly more so with betanaphthol.

There is no apparent reason why the similarity should not extend also to the physiological properties, toxicity to the worms as well as to the host, and, but for small variations, the rationale of the treatment ought to be the same for the two drugs.

#### THE RATIONALE OF BETANAPHTHOL TREATMENT WITH REFERENCE TO THE REMOVAL OF HOOKWORMS AND THE PERCENTAGE OF CURES OBTAINED.

1. *The dosage.* In this part of our investigation our first concern was to ascertain, as definitely as possible, the maximum vermicide dose of betanaphthol to be administered in cases of hookworm infection among adult males. The experiments were accordingly carried out on fifteen batches of adult and apparently healthy male prisoners, who were treated with different dosages of 10, 20, 30, 40, 45, 50 and 60 grains of betanaphthol. Seven groups were given the dose in three portions, three groups received it in two portions, and five groups in one single portion. The drug was administered as a powder, without sugar, and was followed by a mouthful of water. Epsom salts were given as a purge on the evening before treatment, and again two hours after the administration of the last portion of betanaphthol.

In all subsequent treatments 60 grains thymol were used as standard treatment to ascertain the total number of worms in each case.

A 10 grains dosage was fixed upon as the starting submaximal dose with the view to follow the possible toxic symptoms in the host, and the hookworm removal with subsequent increasing dosages.

The stools passed after treatment were collected, washed and examined for five days after the treatment, and the total hookworm content was determined in the usual routine way.

TABLE II.

*Number of hookworms removed by one 'test treatment' of betanaphthol, in three portions (with pre- and after-purge).*

Experiment number.	Test-treatment.	Number of cases treated.	HOOKWORMS REMOVED.			PERCENTAGE OF HOOK- WORMS REMOVED WITH A TEST TREATMENT.			Percentage of cases cured of hookworm infection with a test treatment.	
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.		
1	2	3	4	5			6		7	
1	10 grams in three portions	24	Test-treatment ..	5	118	123	7.1	9.0	8.9	0.0
			Subsequent treat- ments.	65	1,186	1,251				
			Total hookworms	70	1,304	1,374				
2	20 ditto ..	26	Test-treatment ..	29	606	635	42.6	51.4	50.9	11.5
			Subsequent treat- ments.	39	572	611				
			Total hookworms	68	1,178	1,246				
3	30 ditto ..	27	Test-treatment ..	86	684	770	73.5	77.0	77.9	7.4
			Subsequent treat- ments.	31	193	224				
			Total hookworms	117	877	994				
4	40 ditto ..	28	Test-treatment ..	52	682	734	85.2	73.0	75.7	14.3
			Subsequent treat- ments.	9	252	261				
			Total hookworms	61	934	995				
5	40 ditto ..	8	Test-treatment ..	11	363	374	57.8	94.0	92.3	37.4
			Subsequent treat- ments.	8	17	25				
			Total hookworms	19	380	399				
6	50 ditto ..	24	Test-treatment ..	22	526	548	84.6	97.7	97.1	42.8
			Subsequent treat- ments.	4	12	16				
			Total hookworms	26	538	564				
7	60 ditto ..	64	Test-treatment ..	152	2,930	3,082	90.4	97.6	97.2	57.8
			Subsequent treat- ments.	16	70	86				
			Total hookworms	168	3,000	3,168				

TABLE III

*Number of hookworms removed by one 'test-treatment' of helminthol in two portions, at 0, pre- and after-purge).*

Experiment number	Test-treatment	Number of cases treated		HOOKWORMS REMOVED			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST-TREATMENT			Percentage of hookworms removed by one test-treatment
				A. duodenale	N. americanus	A. duodenale and N. americanus	A. duodenale	N. americanus	A. duodenale and N. americanus	
1	2	3	4		5			6		
1	40 2700/100 two portions	20	Test-treatment ..	25	174	199	80.2	89.2	88.8	41.0
			Subsequent treatments	4	21	25				
			Total hookworms	29	195	224				
2	50 3350 ..	50	Test-treatment ..	47	532	579	83.4	91.3	91.6	12.0
			Subsequent treatments	8	31	39				
			Total hookworms	55	563	618				
3	60 ditto ..	30	Test-treatment ..	170	1,717	1,887	91.4	99.9	98.6	60.0
			Subsequent treatments	10	16	26				
			Total hookworms	180	1,733	1,913				

TABLE IV.

*Number of hookworms removed by one 'test treatment' of betanaphthol, in one portion (with pre- and after-purge).*

Experiment number.	Test treatment.	Number of cases treated		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.			Percentage of cases cured of hookworm infection with a test treatment.
				A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.	
1	2	3	4		5			6		7
1	10 grains in one portion.	22	Test treatment ..	18	275	293	15.9	24.2	23.4	4.5
			Subsequent treatments.	95	860	955				
			Total hookworms	113	1,135	1,248				
2	20 ditto ..	23	Test treatment ..	51	523	574	63.7	73.1	72.2	13.0
			Subsequent treatments.	29	192	221				
			Total hookworms	80	715	795				
3	30 ditto ..	32	Test treatment ..	47	763	810	48.7	73.3	71.2	25.0
			Subsequent treatments.	49	278	327				
			Total hookworms	96	1,041	1,137				
4	40 ditto ..	32	Test treatment ..	102	788	890	82.2	93.1	91.7	34.3
			Subsequent treatments.	22	58	80				
			Total hookworms	124	846	970				
5	50 ditto ..	12	Test treatment ..	37	937	974	82.2	98.0	97.3	50.0
			Subsequent treatments.	8	19	27				
			Total hookworms..	45	956	1,001				
6	Betanaphthol No. II. (36 years old). 40 grains in one portion.	46	Test treatment ..	64	819	883	86.5	91.0	90.8	47.8
			Subsequent treatments.	10	80	90				
			Total hookworms..	74	899	973				

The results as figured in Tables II, III and IV show that :—

(a) Whether the drug be administered in one, two or three portions, its anthelmintic effect increases steadily with the dosage.

(b) For the same dosage up to 40 grains, the anthelmintic action is more marked in the case of the one-portion treatment than in the case of the two or three portions treatment.

Beyond 40 grains there is no marked advantage in giving the drug in one massive dose. (Table V.)

TABLE V.

*Cooperative anthelmintic action of 40, 50, and 60 grains dosages of butyrophilol differently administered.*

Experiment number	Test treatment	Number of cases treated		HOOKWORMS REMOVED			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT			Percentage of cases cured of hookworm infestation with a test treatment
				A. diodenale.	N. americanus	A. diodenale and N. americanus.	A. diodenale.	N. americanus	A. diodenale and N. americanus.	
1	2	3	4		5			6		7
1	40 grains in three portions.	28	Test treatment ..	52	682	734	85.2	73.2	75.7	14.3
			Subsequent treatments.	9	252	261				
			Total hookworms.	61	934	995				
2	40 grains in two portions.	20	Test treatment ..	25	174	199	86.2	89.2	88.8	10.6
			Subsequent treatments.	4	21	25				
			Total hookworms.	29	195	224				
3	40 grains in one portion.	32	Test treatment ..	102	788	890	82.2	93.1	91.7	34.3
			Subsequent treatments.	22	58	80				
			Total hookworms.	124	846	970				
4	50 grains in three portions.	21	Test treatment ..	22	526	548	84.6	97.7	97.1	42.8
			Subsequent treatments.	4	12	16				
			Total hookworms.	26	538	564				
5	50 grains in two portions.	50	Test treatment ..	47	532	579	85.4	94.5	93.6	42.0
			Subsequent treatments.	8	31	39				
			Total hookworms.	55	563	618				
6	50 grains in one portion.	12	Test treatment ..	37	937	974	82.2	98.0	95.1	80.0
			Subsequent treatments.	8	19	27				
			Total hookworms.	45	956	1001				
7	60 grains in three portions.	64	Test treatment ..	152	2,930	3,082	90.4	97.6	97.2	97.8
			Subsequent treatments.	16	70	86				
			Total hookworms.	168	3,000	3,168				
8	60 grains in two portions.	36	Test treatment ..	170	1,717	1,887	94.4	99.0	96.7	96.0
			Subsequent treatments.	10	16	26				
			Total hookworms.	180	1,733	1,913				

(c) 60 grains of betanaphthol in two portions may be considered as the maximal dosage in case of hookworm infection among adult males, whether the infection be due to *Ankylostomum duodenale* or *Necator americanus*.

(d) *Ankylostomum* is more betanaphthol-resistant than *Necator*.

2. *Betanaphthol as a vermicide.* Betanaphthol acts as a powerful toxic vermicide for hookworms. The worms expelled are rarely found alive. The dead worms are mostly contorted, but not so much out of shape as is the case with thymol.

Nematodes also yield to betanaphthol in proportion to the strength of the dose, the *Ascaris* being less resistant than *Trichiuris*. Thus 20 per cent *Ascaris* were removed by 30 grains, 10 per cent by 40 grains, 44 per cent by 50 grains, and 62 per cent by 60 grains doses, the infected cases for each group being 5, 10, 16 and 29, respectively. One *Trichiuris* was removed from a case treated with 50 grains, and one from another case treated with 60 grains, although the infection was present in 13 and 21 cases, respectively.

3. *Purging as an aid to betanaphthol treatment.*

(i) All the 186 cases mentioned below were given 40 grains of betanaphthol in one dose. This dosage we knew to be perfectly safe. Moreover as it was somewhat below the optimum dosage, it would lend itself to the study of possible variations due to the purgative.

TABLE VI.

*Anthelmintic action of betanaphthol, with or without purgation.*

Experiment number	Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.			Percentage of cases cured of hookworm infection with a test treatment.
				A. diodendae.	N. americanus.	A. diodendae and N. americanus.	A. diodendae.	N. americanus.	A. diodendae and N. americanus.	
1	2	3	4		5			6		7
H.	40 grains in one portion with a pre- and after purge.	32	Test treatment	102	788	890	82.2	93.1	91.7	34.3
			Subsequent treatments	22	58	80				
			Total hookworms	124	846	970				
b.	40 grains in one portion with no pre- or after purge.	53	Test treatment	51	879	930	79.7	92.1	91.4	47.1
			Subsequent treatments	13	75	88				
			Total hookworms	64	954	1,018				
c.	40 grains in two portions with no pre- or after purge.	50	Test treatment	8	853	861	61.3	87.3	87.0	26.0
			Subsequent treatments	5	124	129				
			Total hookworms	13	977	990				
d.	40 grains in one portion with no purge.	51	Test treatment	12	140	152		7	7	60.8
			Subsequent treatments	2	42	44				
			Total hookworms	14	182	196				
e.	60 grains in two portions with a pre- and after purge.	46	Test treatment	170	1,717	1,887	91.4	99.9	98.6	66.6
			Subsequent treatments	10	16	26				
			Total hookworms	180	1,733	1,913				
f.	60 grains in two portions with no pre- or after purge.	4	Test treatment	45	286	331	84.9	98.5	96.7	91.3
			Subsequent treatments	8	3	11				
			Total hookworms	53	289	342				

(a) In 32 cases the drug was preceded and followed by a purge. All cases had from two to six semi-fluid motions on the first day, but three of them (9 per cent) passed no worms.

(b) 53 cases received no pre-purge; they all had from two to five semi-fluid motions on the first day. With the exception of seven of them (13 per cent), all passed worms.

(c) 50 cases received no after-purge. Of these 46 had from one to three semi-fluid motions on the first day and fourteen (28 per cent) did not pass worms.

(d) 51 cases were treated without purge either before or after treatment, and were given food three hours after the administration of the drug. All, except three, had from one to three motions, semi-solid or semi-fluid. No toxic symptoms immediate or remote due either to the drug or to the absorption of decomposed worms were noticed.

(e) Whatever the mode of treatment, ascarids were often passed on the first day of treatment.

(f) Another series of 44 cases were treated with 60 grains in two doses. The results recorded in Table VI (e), (f), show that the omission of the pre-purge is not attended by any great fall in the efficiency of the drug.

#### 4. *Betanaphthol as a poison to the host.*—

All the dosages we have tried, not excluding the highest of 50 grains in one dose or 60 grains in two doses, are safe. No toxic symptoms were developed; giddiness was rarely noticed, whilst retching and vomiting never occurred.

#### 5. *Efficiency of betanaphthol treatment.*—

Tables II, III and IV, column 7, record the percentages of cures obtained with one test treatment. Notwithstanding the limitations of this method of estimating anthelmintic efficiency, we must admit that the percentages of cures increase with the dosage and with the mode of administration; and that betanaphthol displays marked uniformity in its action.

#### 6. *Discussion of results.*—

(i) Betanaphthol was administered as a powder without sugar.

The drug is usually obtained as a crystalline powder with no tendency to lumping. It need not therefore be powdered anew at the time of administration; nor is mixing with sugar necessary to ensure a particulate condition.

(ii) There is a gradual improvement in efficiency with increase of dosage.

This had already been noted by Nicol<sup>15</sup> in 1912, who experimented with 25, 30, 60 and 90 grains of betanaphthol administered in two or three portions (Table VII).

TABLE VIII.

*Percentage of cures obtained after one treatment with varying dosages of betanaphthol differently administered.*

No.	Authors.	PEOPLE EXPERIMENTED ON.				HOOKWORM INFECTION.			10 GRAINS.		20 GRAINS.		30 GRAINS.		40 GRAINS.			45 GRAINS.	50 GRAINS.			60 GRAINS.		90 GRAINS.	REMARKS.
		Nationality.	Sex.	Age.	Health.	Infection.	Species formula.	Method of diagnosing hookworm or a infection.	One portion.	Three portions.	One portion.	Three portions.	One portion.	Three portions.	One portion.	Two portions.	Three portions.	Three portions.	One portion.	Two portions.	Three portions.	Two portions.	Three portions.	One portion.	
	Elliot, A. G. (1915). <sup>22</sup>	"	"	"	"	"	"	"						58.0											
2	Gonzalez and Luna (1918). <sup>23</sup>	Brazilians.	"	"	"	Heavily infected.	"	"						9 cases.										73.3 100 cases.	No purgative; betanaphthol given 6 grammes once a dose, for three successive mornings; after purge on third morning.  Same as above, but purgative given, and betanaphthol given in one gramme doses every 15 minutes. Total betanaphthol 18 grammes.
3	Riviera and Alvie (1918). <sup>24</sup>	"	"	"	"	"	"	"																85.0 7 cases.	
4	Choudhury Bhaskar (1918-1920). <sup>25</sup>	Tamil Indians.	Males.	A 4 to 11, 20-50 years of age.	Healthy prisoners.	58 hookworms per case.	94 Necator to 6 Ankylostoma.	Howard's centrifuge method; examined 12 or more days after treatment.	4.5 22 cases.	0.0 24 cases.	13.0 23 cases.	11.5 26 cases.	25.0 32 cases.	7.4 27 cases.	34.3 32 cases.	45.0 20 cases.	14.3 24 cases.	37.4 8 cases.	50.0 12 cases.	42.0 20 cases.	42.8 21 cases.	66.6 36 cases.	57.8 64 cases.	—	
	Ditto.	Do.	Do.	Do.	Do.	Do.	Do.	Do.							47.8 46 cases.										Betanaphthol No. 11—70 years old.

TABLE VII.

*Percentage of hookworms removed after one treatment with varying dosages of betanaphthol differently administered.*

(iii) Up to 40 grains, for the same dosage, the one dose treatment is more efficient than the two or three doses treatment.

If the hookworm removal is to be a guide, the one dose treatment has little advantage over the two or three doses treatment when the dosage is increased beyond 40 grains. The worm removal in these cases is so near 100 per cent that small fluctuations are of no account. However, the percentage of cures shows that better results follow from the administration of the drug in one or two portions.

(iv) 50 grains may be taken as the optimum dosage, whether it is administered in one or two or three portions.

Need records a hookworm removal of 97.52 per cent for a 90 grains dosage. Our results with a 50 grains dosage are 97.1, 93.6 and 97.3 per cent.

(v) Whatever the mode of administration, *A. duodenale* is more betanaphthol-resistant than *N. americanus*.

(vi) Betanaphthol is a powerful anthelmintic.

It has been unanimously admitted that it is a potent poison to hookworms.

It is an equally powerful ascariocide. We have already pointed to an *Ascaris* removal of 20, 40, 44 and 62 per cent with dosages of 30, 40, 50 and 60 grains respectively. Schüffner and Vervoot<sup>21</sup> record an *Ascaris* removal of 74 per cent with a dosage of 60 grains. Thus, as is the case with hookworms, the percentage of *Ascaris* removed is proportional to the amount of betanaphthol ingested. This *Ascaris* removal is greater than that obtained with either thymol or chenopodium oil.

(vii) Betanaphthol has a vermifugal action.

The number and nature of the motions obtained in the absence of a purgative show that the drug itself is able to stimulate the intestine and promote evacuation, one of its physiological properties.

Drummond (1910)<sup>11</sup> noted that some coolies ran to diarrhoea after being treated three or four times; and Masfield (1910)<sup>11</sup> found that 20 grains (repeated every day for ten days) caused diarrhoea in some cases (some were sick).

The pre-purge does not appreciably increase the efficiency of the treatment, and may be suppressed altogether from the routine treatment, as is also the case with both thymol and chenopodium oil.

The vermifugal action recorded here raises doubt as to the necessity of the after-purge. Not only did the suppression of the after-purge cause

no toxic symptoms, but the patients, feeling all the better for the absence of the weakening effect of the purge, readily went on with their usual work.

Only 6 per cent of the no purge cases had no motion on the day of treatment; the rest had from one to three motions. Even in the absence of the after-purge the dead hookworms are mostly evacuated on the first day of treatment. (Table IX.)

TABLE IX.

*Percentage of hookworms removed on each day of test treatment with betanaphthol—40 grains in one portion.*

Experiment number.	Test treatment.	Number of cases treated.		1st day of treatment.	2nd day of treatment.	3rd day of treatment.	4th day of treatment.	Total hookworms removed.
a.	With a previous and after-purge.	32	{ Number of hookworms	78.2	10.5	3		890
			{ Percentage to total	87.8	11.8	0.3	—	
b.	With no previous purge	53	{ Number of hookworms	83.1	9.1	7	1	930
			{ Percentage to total	89.4	9.7	0.7	0.1	
c.	With no after-purge	50	{ Number of hookworms	64.3	20.5	6	7	861
			{ Percentage to total	74.7	23.8	0.7	0.8	
d.	With no purge	51	{ Number of hookworms	110	42	0	0	152
			{ Percentage to total	71.9	28.1	—	—	

(viii) Betanaphthol is a safe drug.

The cases of hæmoglobinuria and all the fatal cases recorded in the literature followed upon either the administration of a dose higher than 60 grains or more frequently upon continual administration of the drug for several days in succession <sup>16, 26, 29, 34, 36</sup>

In our cases, no albuminuria, hæmoglobinuria or jaundice was noted; nor did the elimination of the after-purge cause toxæmia.

(ix) Betanaphthol does not deteriorate on ageing.

According to Nicol, betanaphthol is very variable in quality, keeps badly, and should be used in a fresh condition.

We have used during these three years, samples obtained from different sources and have found them chemically pure and equally effective. A sample stored for the past 36 years in our laboratory at Trichinopoly,

was found just as chemically pure, as potent, and quite as safe as any of the new lot. (Table IV, experiment 6.)

#### CONCLUSIONS.

(1) Betanaphthol is a solid drug of constant chemical composition, not deteriorating with age, and easily obtained in the pure state. Its dosage is thus easy and certain.

(2) Betanaphthol is a powerful vermicide acting both on ankylostomes and necators. Up to a dosage of 40 grains, the advantage lies with a single portion treatment; beyond 40 grains, with 50 and 60 grains dosages, the drug may indifferently be given in one, two or three portions.

(3) In sufficiently large doses betanaphthol is an effective ascaricide.

(4) Up to 60 grains dosage betanaphthol is a very safe drug.

(5) Betanaphthol has marked vermifugal properties and no after-purge is required in the treatment.

Treatment with betanaphthol may then be reduced to the simple ingestion of the drug in proper dosage.

The conclusions arrived at for thymol hold good for betanaphthol; and what we have said of the thymol<sup>35</sup> treatment applies equally well to the betanaphthol treatment. We should however state here that, dose for dose, betanaphthol is safer and more efficient than thymol.

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# THE PRODUCTION OF B. INFLUENZÆ VACCINE ON A LARGE SCALE.

BY

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[Received for publication, December 20, 1920.]

VARIOUS authors have described methods of preparing special culture media for B. influenzae which are intended to give an enhanced growth of the organism (Levinthal, Matthews, Liston and Soparkar), or to inhibit certain other associated organisms (Avery). No one, however, seems to have published any method for the production of influenza vaccine in which quantitative results are given, although such vaccine has been largely used during the recent epidemic.

Influenza vaccine is usually prepared on solid media in test tubes, flasks or bottles. This technique, if the vaccine is to be manufactured on a large scale, necessitates a vast amount of labour in the washing and sterilizing of glassware, the preparation of the agar, the sowing (for very generous amounts of seed are required), and the subsequent washing off of the growth; furthermore the large number of units employed (test tubes, flasks or bottles) leads to a multiplication of the necessary controls for purity and sterility of the vaccine.

One way of avoiding this labour would be to grow the organism in broth, using large units, say 2 litre flasks. An objection to this procedure, however, is that the finished vaccine will contain protein material derived from the broth in addition to bacterial protein. In the case of organisms which settle rapidly when grown in broth, this objection need not be considered as the supernatant broth can be removed and the sedimented organisms subsequently suspended in saline and used as antigen for

vaccine or other purposes. Such a method would be economical, and, provided that the antigenic properties of the vaccine are not adversely affected, might well be preferred to other methods where vaccine is to be manufactured on a large scale.

In a study of the biological characters of a large number of strains of *B. influenzae* collected during the recent epidemic, it was found:—

(1) That the great majority of the strains were capable of fermenting glucose to a slight degree, increasing the acidity of a glucose blood broth from PH 7.4 to PH 6 to 6.4.

(2) That in this medium the organisms tended to sediment rapidly leaving behind an almost clear supernatant fluid.

(3) That vigorous growth depended largely on the reaction of the medium employed. The optimum reaction was PH 7.2 to 7.4, while reactions more acid than PH 6.6 or more alkaline than PH 8 resulted in no growth.

(4) That when pigeon blood was used to enrich the culture media the growth was more vigorous than when human, rabbit, sheep, goat or horse blood was used.

(5) That blood heated at 68°C for half an hour was a better enriching fluid than unheated blood.

Advantage has been taken of these facts in devising the method of growing *B. influenzae* described below, and if, as seems probable, acid production beyond a certain hydrogen ion concentration leads to the sedimentation of organisms growing in a fluid culture medium, this method might be applicable to the production of vaccines other than that of *B. influenzae*.

## METHOD.

### I. CULTURE MEDIA.

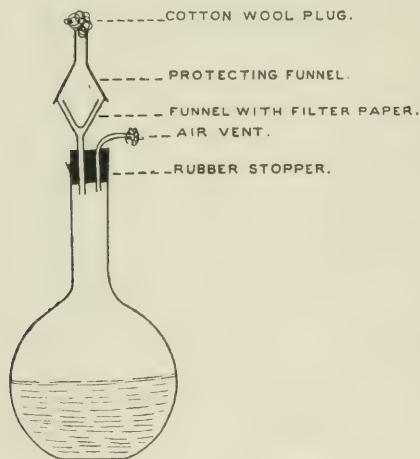
#### (A) *Preparation of Douglas Broth.*

- (1) 1 lb. mutton in 1 litre of tap water.\*
- (2) Add 2 grams of dry sodium carbonate per litre to make faintly alkaline to litmus paper.
- (3) Add 90 c.c. trypsin per litre.†

\* If the meat extract is not to be made into broth in the same day, it is sterilized at 130°C for 1 hour and kept until the following day.

† Trypsin:—1 part sheep's or goat's pancreas, 1 part distilled water, 9 parts distilled water prepared according to the method of Cole and Thomas, *Journal*, Vol. II, 1916, p. 10.

- (4) Incubate at 37°C for 4 hours.
- (5) Filter through muslin.
- (6) Add 0.5 c.c. glacial acetic acid per litre.
- (7) Bring up to 110°C in autoclave to coagulate albumens.
- (8) Filter through cotton wool between two layers of muslin.
- (9) Add 2.5 grams NaCl and 0.125 grams CaCl<sub>2</sub> per litre.\*
- (10) Measure the total amount of broth for the day's brew.
- (11) Adjust the hydrogen ion concentration to PH 7.4.†
- (12) Autoclave at 115°C for 1 hour.
- (13) Filter through muslin and cotton wool.
- (14) Add 1 egg per litre and mix well.



- (15) Autoclave at 130°C for 1 hour to clear.
- (16) Filter through filter paper.

\* The CaCl<sub>2</sub> is added in the form of a watery solution—10 c.c. per litre—in order to save time, as it dissolves rather slowly.

† The hydrogen ion concentration is adjusted by the use of standard tubes prepared and tested by the Medical Research Council, and obtained from Messrs. Baird and Tatlock. PH 7.2 to 7.4 is the optimum hydrogen ion concentration for the growth of *B. influenzae*.

After the final sterilization and addition of extracted blood the hydrogen ion concentration of the broth, ready for use, will be between PH 7.2 to 7.4.

- (17) Fill into 2 litre flasks, 800 c.c. in each flask.\*
- (18) Fit the flasks with rubber stoppers, filter paper and funnels, as shown in the diagram, in order to facilitate the subsequent addition of blood in an aseptic manner.
- (19) Final sterilization in autoclave at 120°C for 1 hour with rubber stoppers, funnels, etc., *in situ*.†

(B) *Collection and addition of blood.*

- (1) Withdraw aseptically 5 c.c. of blood from a pigeon's heart into a syringe containing a few c.c. of 1 per cent sodium citrate in normal salt solution.‡
- (2) Add the blood to a 100 c.c. flask containing 5 grams of glucose dissolved in 50 c.c. of a 1 per cent sodium citrate solution previously sterilized. Mix well.
- (3) Heat in a water-bath at 68°C for half an hour.
- (4) Filter the contents of one small flask directly into each flask containing 800 c.c. of sterile Douglas broth.§
- (5) After filtration is complete, remove the rubber stoppers and funnels, immediately closing each flask with a sterile cotton-wool plug.

The flasks are now ready for sowing. Incubation for 24 hours to test for sterility has been found to be unnecessary.

## II. METHOD OF SOWING.

- (1) Grow the seed (*B. influenzae*) on Douglas agar (PH 7·2—7·4) containing 0·5 per cent glucose and 0·5 per cent heated pigeon blood, for 24 hours at 37°C.||

\* 800 c.c. is used: (1) to facilitate subsequent calculation of the yield of vaccine at 800 millions per c.c.; (2) so that one flask of pigeon blood may be used for each flask of broth. (See under "collection and addition of blood.")

† The sterilization at 130°C (15), followed by the sterilization at the lower temperature of 120°C (18), is necessary to ensure a perfectly clear broth.

‡ By the method described in the *Lancet Med. Research*, Vol. VII, No. 3, p. 503.

§ The filtrate is approximately 40 c.c. and thus the concentration of blood and glucose will each be 0·5 per cent per flask.

|| The broth to be used for making agar is adjusted to PH 7·2—7·4 before the addition of the agar. The agar is ordinary untreated China-grass, having approximately a neutral reaction PH 7·0—7·4. After sterilization and adding blood the resulting agar will be PH 7·2—7·4.

- (2) Sow the flasks by means of the platinum loop.\*
- (3) Incubate the flasks at 37° C for 24 hours.†

### III. COLLECTION OF VACCINE.

After 24 hours incubation the organisms will have settled to the bottom and to some extent along the sides of the flasks, the supernatant broth being clear or very slightly turbid.‡

- (1) Discard obviously contaminated flasks and remove the supernatant fluid from each of the others by means of a suction pump, leaving behind about 30 or 40 c.c. of broth containing the sedimented organisms.
- (2) Test the sediment from each flask for purity by a smear stained by Gram's method, and a sub-culture on plain agar incubated for 24 hours.§
- (3) Add pure carbolic acid to make a concentration of approximately 0·25 per cent.||
- (4) Next day discard all flasks that show impurity either in the stained smear or on the plain agar slope, and pool together the sediments from the remaining flasks.¶
- (5) Test this pooled vaccine for sterility on plain broth (anærobic) and on plain agar (ærobic) incubated for 48 hours.
- (6) Standardise by opacity\*\* and then add pure carbolic acid to make a concentration of 0·5 per cent.††
- (7) Store vaccine which has passed the sterility test until needed for bottling.
- (8) When ready for bottling, dilute to the required strength with sterile normal salt solution.

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\* Contrast this with the large amount of seed required in the "bottle method," viz., the entire growth of one agar slope per bottle.

† It is important to remember that temperatures over 41°C will kill *B. influenzae*.

‡ A turbid supernatant fluid and a change in the colour of the broth are signs of contamination.

§ There usually is a very fine filmy growth of *B. influenzae* on the plain agar due, no doubt, to a small amount of blood broth brought over by the platinum loop. If there is any doubt regarding the origin of this growth a smear should be made and stained by Gram's method.

|| This is sufficient to sterilize the vaccine: heat is not required.

¶ The sediment consists entirely of bacteria; there is no extraneous deposit as far as one can judge from stained specimens.

\*\* Brown. *Ind. Jour. Med. Research*, Vol. VII, No. 1, July, 1919, p. 238.

†† Carbolic acid in concentrations greater than 0·5 per cent appreciably increase the turbidity of the vaccine, and so care must be taken to avoid excess of carbolic before the vaccine has been standardized.

## YIELD OF VACCINE.\*

Strain of B. influenza.	Origin of strain.	No. of flasks from which vaccine was obtained.	YIELD OF VACCINE AT 800 MILLIONS ORGANISMS PER C.C.	
			Total no. of c.c.	Average no. of c.c. per flask †
Dharma ..	Isolated from sputum of influenza patient, April, 1920 .. ..	5	3,834	767
Hita ..	Isolated from sputum of influenza patient, Octo- ber, 1918 .. ..	42	39,183	933
R. R. ..	Isolated from sputum of influenza patient, April, 1920 .. ..	8	46,527	970
C. 200 ..	Isolated from sphenoidal sinus of influenza patient, post-mortem, March, 1919 .. ..	73	68,564	939
Total‡ ..	....	168	158,108	941

\* Figures taken from the records of the Central Research Institute, Kasauli, representing the yield for the first 25 working days after this method was adopted as a routine.

† These figures also represent the average yield in millions of organisms per c.c. of broth.

‡ During this period 255 flasks were prepared, and 87.64 per cent. were rejected on account of failure to pass the purity and sterility tests.

*Table showing the mortality among the pigeons used to supply blood for B. influenza culture media from October 10th to December 1st, 1920.*

				Deaths due to bleeding.	Mortality per cent.
Number of pigeons bled once	..	..	145	10	6.9
.. .. .	..	twice	85	6	7.0
.. .. .	..	three times	70	2	2.8
.. .. .	..	four times	63	4	6.3
.. .. .	..	five times	50	1	2.0
.. .. .	..	six times	48	4	8.3
.. .. .	..	seven times	44	4	9.1
.. .. .	..	eight times	28	2	7.1
.. .. .	..	nine times	4	1	25.0
Total number of bleedings	..	..	539	34	6.3

*Deaths due to causes other than bleeding = 8.*

*Notes.*—Five c.c. of blood were withdrawn from each pigeon on each occasion. This is about the maximum amount which can be rapidly withdrawn from the heart, with safety, at a single bleeding. An interval of five or six days was allowed between successive bleedings.

The bleedings were performed for the most part by one laboratory attendant who had had no previous experience in this work.

Leaving out the figures for the ninth bleeding, when only 4 pigeons were used, it will be seen that the mortality is very low, and increases slightly, if at all, with frequency of bleeding.

#### CONCLUSIONS.

(1) *B. influenza* vaccine can be manufactured on a large scale by growing the organism in Douglas mutton broth (PH 7.2 to 7.4) containing 0.5 per cent glucose and 0.5 per cent heated pigeon blood.

(2) With the procedure described above the final product is a suspension of organisms in normal salt solution, practically free from foreign protein derived from the broth.

(3) The method is economical in time and labour and may be expected to give a yield of 900 to 1000 millions of organisms per c.c. of broth.

(4) The method should be applicable to vaccine production where organisms other than *B. influenza* are concerned.

# A NOTE ON THE LARGE SCALE PRODUCTION OF PNEUMOCOCCUS VACCINE.

BY

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[Received for publication, February 21, 1921.]

IN another communication<sup>1</sup> I showed that *B. influenzae* vaccine could be rapidly produced by growing the organism in tryptic-digest mutton broth (Douglas), PH 7.2 to 7.4, containing 0.5 per cent glucose and 0.5 per cent heated pigeon blood.

The strains of *B. influenzae* used were isolated from cases of influenza during an epidemic period. They fermented glucose with production of acid (PH 5 to 6.4), and sedimented readily after 24 hours growth in the culture medium employed. The vaccine was made by removing the clear supernatant broth, and suspending the sedimented organisms in normal salt solution.

It is probable that the sedimentation of the organisms is largely due to the increased acidity of the broth following the fermentation of the glucose present. If this be so, the method should be applicable to vaccine production where organisms other than *B. influenzae* are concerned.

This short note gives the results of the application of the method to the large scale production of pneumococcus vaccine.

*Pneumococcus* ferments glucose with production of acid (PH 5 to 5.6), and grows well in tryptic-digest broth containing heated pigeon blood, and so an exactly similar culture medium was used for this vaccine as for the *B. influenzae* vaccine.

## METHOD.

The various steps in the method are as follows :

- (1) Prepare a series of litre flasks each containing 500 c.c. of glucose blood broth as for *B. influenzae* vaccine.

- (2) After adding the blood incubate the flasks at 37°C, and reject any that show signs of contamination at the end of 24 hours.
- (3) Use, as seed, a 24-hour growth of pneumococcus on ordinary rabbit blood agar, PH 7.2 to 7.4, and sow each flask by means of a platinum loop.
- (4) Incubate the cultures for 72 hours at 37°C so as to ensure good sedimentation. (*Note*.—The sedimentation is not as complete as in the case of *B. influenzae*, and the supernatant fluid is always slightly turbid.)
- (5) At the end of this period remove the supernatant fluid by means of a suction pump.
- (6) Test the sediment of each flask for purity by subculture on plain agar incubated at 37°C for 24 hours.
- (7) Add sterile normal salt solution to each flask to make up to 500 c.c.
- (8) Add 0.5 per cent phenol, and allow to stand at room temperature overnight.
- (9) Reject all flasks that fail to pass the purity test, and test the remainder for sterility by subculture on plain broth (anaerobic) and on plain agar (aerobic) incubated for 48 hours.
- (10) Reject contaminated flasks. Standardize the vaccine in the sterile flasks by opacity,<sup>2</sup> and store until needed for bottling.

*Yield of Vaccine.*

Strain of <i>Pneumococcus</i> .	Origin of Strain.	Number of flasks from which vaccine was obtained.	Average yield of vac- cine expressed as millions of organisms per c.c. of broth.
Ks. Type I ..	Sputum of fatal case of lobar pneumonia (Kasauli).	92	2990
L. Type II ..	The National Collec- tion of Type Cultures, Lister Institute.	70	2380
		162	2728

NOTES :—(1) The figures are taken from the records of the Central Research Institute, Kasauli, and represent the yield of vaccine for the first twenty working days after this method was adopted as a routine.

(2) During this period 227 flasks, each containing 500 c.c. of broth were sown, and 65 (29 per cent) were rejected on account of failure to pass the purity and sterility tests.

## REFERENCES.

- <sup>1</sup> MALONE .. .. The production of B. influenza Vaccine on a large scale.  
*Indian Journal of Medical Research*, (this number).
- <sup>2</sup> BROWN .. .. Further observations on the standardization of bacterial  
suspensions. *Indian Journal of Medical Research*,  
1919, Vol. VII, No. 1, p. 238.

# BACTERIOLOGICAL AND LABORATORY TECHNIQUE

## Section II.

BY

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[Received for publication, January 7, 1921.]

### MEDIA.

#### 1. MEDIA. PREPARATION OF AGAR : M1·1 FIBRE AGAR :

**M1·11.**—(1) Prepare :—Fibre agar 1 ; glacial acetic acid 0·05 ; water 2. (2) Allow the agar to soak in the acidified water 15 min. (3) Remove the fibre agar and wash thoroughly with water until quite free from any trace of acid reaction to litmus paper. (4) Squeeze in a cloth to get rid of excess of water. (5) Use in the preparation of media, to give a 3 per cent sol. of agar, calculating from the weight of the agar in the original dry condition.

**M1·12.** (1) Prepare :—Fibre agar 10 ; bouillon<sup>1</sup> (**M2·111**) 500. (2) Allow to soak 2 hr. (3) Bring into sol. with heat. (4) Convert into agar bouillon (**M2·13**).

**Note.**—<sup>1</sup>Instead of using bouillon the fibre agar may be allowed to soak overnight in water and the water removed in the morning.

**M1·13.**—(1) Prepare :—Fibre agar 10 ; water 500. (2) Dissolve with heat. (3) Filter. (4) Add the filtrate, made up to a volume of 500 c.c., to bouillon<sup>1</sup> (**M2·111**) of double strength.

**Note.**—<sup>1</sup>That is to say, bouillon made with 500 grm. meat to 500 c.c. water and containing the same quantity of peptone and sod. chloride as is used for 1000 c.c. ordinary bouillon.

**M1·2 POWDERED AGAR : M1·21.** (1) Use in weighed quantities 1·5 to 2 per cent. by simple addition<sup>1</sup> directly to the meat extract in the same way (**M2·111**) as peptone and sod. chloride are added.

**Note.**—<sup>1</sup>The powdered agar should be made up into a paste or suspension with a little meat extract before addition to the remainder of the meat extract.

**M1'22.** (1) Take the fibre agar as prepared in **M1'11**. (2) Place the fibre on a filter funnel furnished with moistened cotton wool<sup>1</sup> for filtration. (3) Place the filter funnel with agar in an autoclave over a collecting receptacle. (4) Keep 1 hr. at 120°C. (5) Cool rapidly the receptacle, now containing the cleaned filtered agar. (6) Remove the agar when set. (7) Mince finely by passing the agar through a mincing machine furnished with a disc having small holes. (8) Spread the vermicular threads obtained in shallow layer on trays. (9) Desiccate in a drying oven at moderate temperature. (10) Reduce the desiccated material to powder. (11) Preserve in the dry state. (12) Use for the preparation<sup>2</sup> of media in a strength of 1 per cent.

**Notes.** —<sup>1</sup>The filtering wool is supported on butter muslin and the free edges of the latter are turned over the rim of the funnel and tied there. This prevents the wool from sinking down into the narrow part of the funnel and blocking the filtration. —<sup>2</sup>The desiccated agar is brown in appearance but becomes white and transparent when brought into solution. As it has been filtered and cleaned it only requires to be weighed and added direct to a fluid medium. No further filtration is necessary.

## 2. MEDIA. PREPARATION AND COMPOSITION: M2'1 MEAT EXTRACT -PEPTONE.<sup>1</sup>

**Notes.**—<sup>1</sup>Syn. nutrient bouillon, bouillon, broth. The term bouillon will be used to signify meat extract peptone fluid medium, and where this term is used without any qualification it refers to the method **M2'111**.

**M2'11 BOUILLON: M2'111.**<sup>1</sup>—(1) Mince finely fat-free beef.<sup>2</sup> (2) Add 500 gm. to 1000 c.c. D. W. or clear T. W. (3) Heat<sup>3</sup> the mixture 20 min. over a free flame, at a temperature not exceeding 50°C. (4) Skim off fat floating on the surface. (5) Raise the temperature to boiling point. (6) Boil 10 min. (7) Pour the mixture on to a wet, thick, clean cloth. (8) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (9) Filter the fluid collected through well-wetted, thick filter paper. (10) Add<sup>4</sup> to the filtrate: peptone<sup>5</sup> 10 gm.; sod. chloride 5 gm. (11) Steam or boil 45 min. (12) Bring the vol. up to 1000 c.c. by the addition of water. (13) Estimate and adjust<sup>6</sup> (**S7'76, 7'8**) the reaction of the medium. (14) Steam<sup>7</sup> 30 min. (15) Clear.<sup>8</sup> If necessary, and while hot, through well-wetted, thick filter paper, or through two layers of absorbent cotton wool. (16) Distribute the filtrate into flask or T. T. (17) Sterilize.

**Notes.** —<sup>1</sup>The description here given will be taken as the standard formula for media. —<sup>2</sup>Wool, chicken, ox or horse meat, horse blood, mutton or pig blood, pancreas, liver, spleen, kidneys, brain, and vegetable materials, such as yeast, are also used. —<sup>3</sup>Heat

serve to furnish the extract used as basis for the medium. <sup>3</sup>Or simply keep in a cool place overnight. <sup>4</sup>The peptone should be worked into a paste or suspension by gradual addition of a little of the meat extract before addition to the filtrate. <sup>5</sup>A still higher percentage of peptone is sometimes advantageous. Care must be taken to select a good standard bacteriological peptone. Such a peptone should be completely soluble with heating in water, should not contain fermentable sugar, and should afford a satisfactory pabulum for micro-organisms. Commercial "peptones" differ in regard to their composition, amino-acid content, etc., and in regard to suitability for certain special tests such as the indol test. <sup>6</sup>A very generally used adjustment for ordinary laboratory use is to make the reaction faintly alkaline to litmus, or 1 per cent (S7·81) acid to phenolphthalein. The standard method of adjustment is by hydrogenion concentration (S7·83 to 7·86). <sup>7</sup>To stabilize the reaction and so precipitate earthy phosphates. This precipitation may also be effected by heating 5 min. at 116 C. If such a high temperature is used, the subsequent sterilization must be performed, if precipitation in the medium is to be avoided, at a somewhat lower temperature. <sup>8</sup>If simple filtration through thick paper alone is not sufficient to give a clear medium, clearing should be effected by means of white of egg or other clearing agent (M5·1).

**M2·112.**<sup>1</sup>—(1) Mince finely fat-free beef. (2) Add 500 grm. to 1000 c.c. D. W. or T. W. (3) Keep in a cool place overnight. (4) Skim off fat floating on the surface. (5) Pour the mixture on to a wet, thick, clean cloth. (6) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (7) Bring the volume up to 1000 c.c. by the addition of water. (8) Add peptone 10 grm.; sod. chloride 5 grm. (9) Estimate and adjust (S7·76, 7·8) the reaction of the medium. (10) Steam or boil 45 min. (11) Filter, while hot, through well-wetted, thick, filter paper or through two layers of absorbent cotton wool. (12) Distribute the filtrate into flasks or T. T. (13) Sterilize (S9·5, 9·6).

**Notes.**—<sup>1</sup>Consult notes M2·111 for explanatory detail. <sup>2</sup>The albuminous material precipitated out of the meat extract takes the place of the ordinary white of egg added for clarification purposes.

**M2·113 LEMCO.** (1) Add the whites of 2 eggs to 1000 c.c. water. (2) Add to the mixture by degrees to make a suspension: lemco<sup>1</sup> 3 to 5 grm.; peptone 10 grm.; sod. chloride 5 grm. (3) Steam or boil 45 min. (4) Filter, while hot, through well-wetted, thick, filter paper or through two layers of absorbent cotton wool. (5) Bring the vol. up to 1000 c.c. by the addition of water. (6) Estimate and adjust (S7·576, 7·8) the reaction of the medium, if necessary.<sup>2</sup> (7) Sterilize (S9·5, 9·6).

**Notes.**—<sup>1</sup>To take the place of meat extract obtained from fresh meat. The results in respect of growth of organisms are not so good as when fresh meat extract is used. The medium is practically a sugar-free medium. <sup>2</sup>Adjustment of reaction, except to give a specially required reaction, is unnecessary.

**M2-114 ACID DIGEST MEDIUM.**—(1) Mince finely 1 lb. free goat, sheep or ox flesh. (2) Add to it 80 c.c. strong commercial hydrochloric acid, of specific gravity 1.16, per kilogramme. (3) Mix thoroughly. (4) Keep in a water bath 1 d. at 70 C. (5) Bring the volume up to 1000 c.c. for each kilogramme of minced meat used. (6) Prepare acid digest of meat 1 : boiling water 2. (7) Add 75 c.c. 40 per cent sod. hydroxide for each kilogramme of minced meat used. (8) Make the reaction faintly alkaline to litmus. (9) Steam 60 min. (10) Pour the mixture on to a wet, thick, clean cloth. (11) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (12) Estimate and adjust the reaction of the medium (**S7-76, 7-8**). (13) Filter through well-wetted, thick, filter paper. (14) Prepare —filtrate 1 ; boiling water 2. (15) Sterilize (**S9-8**) in the autoclave.

**M2-115<sup>1</sup> LIVER.<sup>2</sup>** (1) Make a sterilized liver extract in the same way as a meat extract with 1000 gm. finely minced fresh ox liver and 1000 c.c. water. (2) Make a solution,<sup>3</sup> peptone 2 per cent ; sod. chloride 1 per cent. (3) Sterilize the peptone sol. (4) Prepare, while the solutions are hot, and with sterile precautions : —sterilized liver extract 1 ; sterilized peptone sol. 1. (5) Distribute with sterile precautions into T. T.

**Notes.**—<sup>1</sup>No adjustment of reaction is made. For cultivation of meningococci, etc., when solidified with agar. <sup>2</sup>Other organs, placenta, etc. may be similarly treated. <sup>3</sup>The solution may contain 3 per cent agar, if a solid medium is to be made.

**M2-116<sup>1</sup> BLOOD.**—(1) Allow fresh ox blood to clot. (2) Place in an ice chest 3 hr. (3) Detach the clot. (4) Remove it and mince finely. (5) Replace the clot in the serum. (6) Weigh clot and serum together. (7) Add twice the weight of D. W. (8) Raise the temperature to boiling point, with constant stirring. (9) Boil 10 min. (10) Pour the mixture on to a wet, thick, clean cloth. (11) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (12) Boil the fluid collected. (13) Skim off the coagulated proteid as it collects on the surface. (14) Add 0.5 c.c. glacial acetic acid per litre. (15) Filter through well-wetted, thick, filter paper. (16) Add to the filtrate :—peptone 1 per cent ; sod. chloride 0.5 per cent. (17) Steam or boil 45 min. (18) Adjust the reaction (**S7-76, 7-8**) (19) Distribute into flasks or T. T. (20) Sterilize (**S9-5, 9-6**)

**Note.**—<sup>1</sup>An almost sugar-free medium. It may be solidified with agar (**M2-131**).

**M2-117 FISH.** (1) Mince finely the flesh of cod. (2) Add 500 gm. to the following sol. :—sod. chloride 20.5 gm., pot. chloride

0.75 gm.; mag. chloride 3.25 gm.; water 500 c.c. (3) Heat the mixture 20 min. over a free flame at a temperature not exceeding 50 C. (4) Raise the temperature to boiling point. (5) Boil 10 min. (6) Pour the mixture on to a thick, clean cloth. (7) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (8) Filter the fluid collected through well-wetted, thick, filter paper (9) Add peptone 5 gm. (10) Steam 45 min. (11) Bring the vol. up to 1000 c.c. by the addition of water. (12) Steam 30 min. (13) Filter, while hot, through well-wetted, thick, filter paper. (14) Distribute into flasks or T. T. (15) Sterilize (S9.5, 9.6).

**M2.12 GELATIN BOUILLON.**<sup>1</sup>—(1) Proceed as in the preparation of bouillon (M2.111) through steps (1) to (8). (2) Add<sup>2</sup>:—peptone 10 gm.; sod. chloride 5 gm.; gelatin<sup>3</sup> 120 gm. (3) Heat gently with constant stirring. (4) Steam or boil 45 min. to obtain complete solution. (5) Bring the vol. up to 1000 c.c. by the addition of hot<sup>4</sup> water. (6) Estimate and adjust the reaction of the medium (S7.76, 7.8). (7) Steam<sup>5</sup> 30 min. (8) Filter,<sup>6</sup> while hot, through well-wetted, thick, filter paper by placing filter funnel, stand, and receptacle for filtrate in the steam sterilizer, and steaming till filtration is complete. (9) Distribute<sup>7</sup> into flasks or T. T. (10) Sterilize (S9.5, 9.6). (11) Cool the flasks or T. T. on final removal from the sterilizer. (12) Store in a cool place.

**Notes.**—<sup>1</sup>Any fluid medium may be similarly solidified with gelatin. <sup>2</sup>The gelatin may be added to bouillon (M2.111) if that is ready prepared. <sup>3</sup>The gelatin, which should be the best French "gold label" gelatin or one of equivalent purity, is cut up into small pieces and added to the meat extract. <sup>4</sup>The solution must be kept warm to prevent solidification, until ready for filtration. <sup>5</sup>To precipitate earthy phosphates. <sup>6</sup>Filtration may be effected by using a hot water filter funnel. If filtration through paper is not sufficient to give a clean, medium clarification should be effected by means of white of egg or other clearing agent (M5.1). <sup>7</sup>Use a small filter funnel with rubber attachment and clamp for the purpose.

### **M2.13 AGAR BOUILLON.**<sup>1</sup>

**Note.**—<sup>1</sup>Any fluid medium may be solidified with agar. Syn. "nutrient agar" to be distinguished from "agar" the powder or fibre, and "agar sol." which is a sol. of agar in water. When the term "nutrient agar" is used without qualification it refers to the product M2.131.

**M2.131.**—(1) Proceed as in the preparation of bouillon (M2.111) through steps (1) to (8). (2) Add per 1000 c.c. of fluid obtained:—peptone 10 gm.; sod. chloride 5 gm.; prepared agar (M1.11) 30 gm.<sup>1</sup> (3) Steam gently 2½ hr.<sup>2</sup> to bring the agar thoroughly into solution. (4) Bring the vol. up to 1000 c.c. by the addition of hot water.<sup>3</sup>

(5) Estimate and adjust the reaction of the medium (S7·76, 7·8). (6) Steam<sup>4</sup> 30 min. (7)<sup>5</sup> Filter, while hot, through well-wetted, thick, filter paper or two layers of absorbent cotton wool, by placing filter funnel, stand, and receptacle for filtrate in the steam sterilizer and steaming till filtration is completed. (8) Distribute into flasks or T. T. (9) Sterilize (S9·5, 9·6).

**Notes.** <sup>1</sup>If powdered agar is used, 20 gm. per 1000 c.c. of medium is sufficient. <sup>2</sup>Or heat 45 min. at 118 C. <sup>3</sup>The solution must be kept hot to prevent solidification until ready for filtration and sterilization. Agar goes into solution at about 92 C. and solidifies at a temperature of about 40 C. <sup>4</sup>To precipitate earthy phosphates. <sup>5</sup>If filtration through paper is not sufficient to give a clear medium, clarify with white of egg or other clearing agent (M5·1).

**M2·132.**—(1) Cut up 20 gm. fibre agar<sup>1</sup> into small pieces. (2) Add to 1000 c.c. hot bouillon (M2·111). (3) Bring the agar into solution with heat after having been allowed to soak 2 hr. in the hot bouillon. (4) Estimate and adjust the reaction of the medium (S7·76, 7·8). (5) Steam 30 min. (6) Clarify and filter (M5·1). (7) Distribute into T. T. (8) Sterilize (S9·5, 9·6).

**Note.**—<sup>1</sup>Agar may be obtained in powder form<sup>2</sup> (M1·21) and may be used in place of fibre agar.

**M2·133.**—(1) Place 15 gm. fibre agar in 500 c.c. T. W. (2) Wash the agar well by squeezing it through the hands. (3) Decant and reject the dirty water. (4) Replace with the same amount of clean T. W. (5) Heat over a free flame with constant stirring to dissolve the agar. (6) Add peptone 5 gm.; sod. chloride 2·5 gm. (7) Add to 500 c.c. meat extract (M3·61) 10 gm. egg albumin which has been made into a paste or suspension with a little of the meat extract. (8) Add the dissolved agar slowly to the meat extract thus prepared, with constant stirring. (9) Steam 60 min. or heat 45 min. at 120 C. (10) Bring the vol. to 1000 c.c. by addition of water. (11) Estimate and adjust the reaction. (12) Boil 15 min. over a free flame. (13) Make up for any loss of volume by addition of water. (14) Filter, while hot, through well-wetted, thick, filter paper by placing filter funnel, stand, and receptacle for filtrate in the steamer and steaming till filtration is completed. (15) Distribute filtrate into flask or T. T. (16) Sterilize (S9·5, 9·6).

**M2·14 GELATIN AGAR MEDIUM : M2·141<sup>1</sup>** (1) Use gelatin 10 per cent plus agar 0·5 per cent to solidify bouillon (M2·111).

**Note.**—<sup>1</sup>A medium usable at incubation temperature of 30 C.

**M2·142.<sup>1</sup>**—(1) Use gelatin 12 per cent plus agar 0·5 per cent to solidify bouillon (M2·111).

**Note.**—<sup>1</sup>A medium usable at incubation temperature of 37 C.

**M2·2 BLOOD : M2·21 WHOLE BLOOD : M2·211.**—(1) Wash and scrub the finger to be used well with hot soap and water. (2) Drop on to the finger abs. alc. followed by ether to remove the alc. (3) Congest the pulp of the finger by winding a bandage round the base. (4) Prick the congested pulp with a sterile needle. (5) Take up a drop of the blood which exudes in a sterile platinum loop. (6) Smear<sup>1</sup> on the surface of an agar slope. (7) Cover the T. T. with an india-rubber cap. (8) Test sterility before use by incubation 48 hr.

**Note.**—<sup>1</sup>Serum may be used in the same way.

**M2·212.**<sup>1</sup>—(1) Add 0·25 c.c. blood to 5 c.c. melted agar in a T. T. (2) Boil 1 min. (3) Slope.<sup>2</sup>

**Notes.**—<sup>1</sup>For cultivation of *B. influenzae*. <sup>2</sup>The precipitate settles to the bottom.

**M2·213.**<sup>1</sup> (1) Use meat medium (**M3·51**) to which a little whole blood has been added.

**Note.**—<sup>1</sup>For stock culture *B. influenzae*, etc. The medium is capable of maintaining the viability of *B. influenzae* 6 wk.

**M2·214.**—(1) Add directly, blood drawn off from a vein<sup>1</sup> or from the heart with a sterile syringe to melted nutrient agar in a T. T. at 45 C.<sup>2</sup> (2) Roll the T. T. between the palms to mix. (3) Slope. (4) Test sterility by incubation 48 hr.

**Notes.**—<sup>1</sup>Man, sheep, rabbit, etc. <sup>2</sup>The temperature of the agar should not be too high else the blood will settle down from the surface of the blood agar slope while it is solidifying.

**M2·215.**<sup>1</sup>—(1) Add 15 c.c. aspirated malarial blood to a centrifuge tube containing 0·1 c.c. 50 per cent glucose. (2) Defibrinate the blood-glucose mixture with a glass rod. (3) Centrifuge. (4) Observe the development<sup>2</sup> of parasites at 41 C. under anaerobic conditions.

**Notes.**—<sup>1</sup>For cultivation malaria parasites. <sup>2</sup>The parasites develop in the layer 1·50th to 1·20th in. from the top of the deposit. All the parasites in the deeper lying red cells die. Should the cultivation of more than one generation be desired the leucocyte upper layer must be carefully pipetted off, as the leucocytes immediately destroy the merozoites. Only the parasites within the erythrocytes escape phagocytosis.

**M2·216.**<sup>1</sup>—(1) Prepare agar sol. :—sod. chloride 6, well washed agar 16; water 900. (2) Sterilize. (3) Prepare :— rabbit blood 1; agar sol. at 50 C. 3. (4) Slope. (5) Test sterility. (6) Preserve in the dark.

**Note.**—<sup>1</sup>For cultivation of *Leishmania*.

**M2·22 DEFIBRINATED BLOOD : M2·221.**<sup>1</sup> (1) Defibrinate human or rabbit blood. (2) Add 10 times its vol. D. W. (3) Add

1 vol. of the laked blood thus obtained to 2 vol. 1·2 per cent sod. chloride.

**Note.**—<sup>1</sup>For cultivation of *Leishmania*.

**M2·222.**<sup>1</sup>—(1) Prepare : sterilized<sup>2</sup> defibrinated human blood<sup>3</sup> at 45 C., 5 drops ; melted nutrient agar<sup>4</sup> 0·6 per cent acid to phenolphthalein at 45 C.

**Notes.**—<sup>1</sup>For the cultivation of the gonococcus. <sup>2</sup>Sterilize 8 d. at 57 C. <sup>3</sup>Serum may be used instead of blood. <sup>4</sup>Preferably nutrient agar in which sod. chloride is replaced by disod. phosphate. The substitution of phosphate is important.

**M2·223.** (1) Prepare : sterile defibrinated blood at 45 C. 1 ; melted nutrient agar at 45 C. 2.

**M2·224.**<sup>1</sup>—(1) Prepare : potato extract (**M4·813**) 10 ; 0·6 per cent sod. chloride 30 ; agar 1. (2) Sterilize. (3) Distribute in T. T. (4) Prepare with sterile precautions : melted potato extract agar at 45 C. 1 ; sterile defibrinated blood at 45 C. 1. (5) Test sterility by incubation 48 hr.

**Note.**—<sup>1</sup>For the cultivation of delicate organisms, such as the influenza bacillus, *B. pertussis*, meningococcus, gonococcus, etc.

**M2·225.**<sup>1</sup>—(1) Prepare alkali blood : defibrinated ox blood 1 ; N-1 sod. hydroxide<sup>2</sup> 1. (2) Sterilize (**S9·5**) 3 d. at 100 C. (3) Prepare : sterilized alkali blood at 45 C. 3 ; melted nutrient agar neutral to litmus at 45 C. 7. (4) Distribute<sup>3</sup> immediately into T. T. or plates.

**Notes.**—<sup>1</sup>For isolation of *V. cholerae*. <sup>2</sup>Or 11·4 per cent anhydrous sod. carbonate. <sup>3</sup>Should be kept freely open after tubing or plating, under sterile sheets of paper, for 48 hr. at 37 C., or under a bell jar containing carbon dioxide gas for 30 to 60 min.

**M2·226.**<sup>1</sup>—(1) Prepare :—melted nutrient agar containing 2 per cent peptone at 45 C. 1 ; sterile defibrinated rabbit blood at 45 C. 2.

**Note.**—<sup>1</sup>For cultivation of *Leishmania*, etc.

**M2·227.**—(1) Collect blood aseptically in a sterile flask containing glass beads. (2) Shake 10 min. (3) Distribute with sterile precautions into T. T. (4) Test sterility by incubation 48 hr.

**M2·228.**—(1) Prepare :—defibrinated ox blood 1 ; N-1 sod. hydroxide 1. (2) Steam 60 min. (3) Desiccate *in vacuo* over sulphuric acid or calc. chloride. (4) Grind the residue to powder. (5) Make for use a 10 per cent solution of blood powder. (6) Prepare : 10 per cent blood powder solution at 45 C. 3 ; melted nutrient agar at 45 C. 7.

**M2·229.**<sup>1</sup>—(1) Prepare :—lactose 0·3 ; melted nutrient agar 10. (2) Raise slowly to boiling water temperature. (3) Cool to 45 C.

(4) Add 1 c.c. defibrinated sterile human blood at 45 C., and 3 c.c. 1 per cent alc. rosolic acid at 45 C. (5) Prepare plates. (6) Keep 2 d. before use.

**Notes.**—<sup>1</sup>For cultivation of typhoid, coli, dysentery organisms, etc.

**M2·23 DEFIBRINATED BLOOD: M2·231<sup>1</sup>.**—(1) Prepare :—fibre agar 20; T. W. 1000. (2) Dissolve the agar. (3) Distribute in T. T. (4) Sterilize. (5) Prepare:—sterilized agar sol. 1; sterile defibrinated rabbit blood 1. (6) Solidify in the sloped position. (7) Sow the organism in the water of condensation.

**Notes.**—<sup>1</sup>For the cultivation of trypanosomes of cold-blooded animals.

**M2·232.<sup>1</sup>** (1) Prepare blood fluid:—defibrinated blood 1; D. W. 3. (2) Add 0·5 c.c. 10 per cent sod. hydroxide<sup>2</sup> per 100 c.c. blood fluid. (3) Sterilize (S9·6) in the autoclave at 112 C. (4) Prepare:—sterilized blood fluid<sup>3</sup> at 80 C.<sup>4</sup> 1; melted pepsin digest agar (M3·9211) at 80 C., 2. (5) Slope.

**Note.**—<sup>1</sup>For cultivation of meningo coccus, etc. <sup>2</sup>The addition of alkali permits of sterilization without coagulation. <sup>3</sup>The blood fluid may be rendered only just alkaline to litmus by the addition of hydrochloric acid before addition to the agar. <sup>4</sup>In order to avoid precipitation of colloids.

**M2·233.<sup>1</sup>**—(1) Prepare peptone agar:—peptone 0·5; sod. chloride 1; agar 1·5; water 100. (2) Prepare:—melted peptone agar at 45 C. 1; defibrinated rabbit's blood at 45 C., 1.

**Note.**—<sup>1</sup>For cultivation of trypanosomes, etc.

**M2·234.<sup>1</sup>**—(1) Prepare agar sol.:—sod. chloride 0·6; agar 1·5; water 100. (2) Prepare:—melted agar sol. at 45 C., 1; defibrinated rabbit's blood at 45 C., 1.

**Note.**—<sup>1</sup>For cultivation of trypanosomes, etc.

**M2·235.<sup>1</sup>**—(1) Prepare:—defibrinated rabbit or human blood 1; melted nutrient agar at 70 C., 20. (2) Raise the temperature of the mixture to boiling point over a free flame. (3) Shake to mix. (4) Raise to boiling point twice again. (5) Allow to deposit. (6) Decant the clear S. N. F. into T. T. while the agar is still melted, or filter through glass wool. (7) Slope.

**Note.**—<sup>1</sup>For cultivation of B. influenzae, etc.

**M2·236.** (1) Prepare:—stock "tryptamine" medium (M4·732) 1; water 2. (2) Make distinctly acid to litmus by the cautious addition of strong hydrochloric acid. (3) Add agar 2 per cent. (4) Steam 2½ hr. (5) Filter, while hot, through well-wetted, thick, filter paper by placing filter funnel, stand, and receptacle for filtrate in the steamer and steaming

gill filtration is completed. (6) Add per litre of melted agar at 65 C. 50 c.c. defibrinated sheep's blood and the beaten up white of 2 eggs. (7) Steam 50 min. (8) Strain the mixture through a fine wire sieve and squeeze<sup>1</sup> the fluid out of the clot. (9) Filter through glass wool, taking care to keep the mixture hot during the process. (10) Adjust the reaction. (11) Distribute in T. T. (12) Sterilize 15 min. at 100 C. 3/4.

**Note.**—<sup>1</sup>With a glass plate or clock glass.

**M2:237.**<sup>1</sup>—(1) **Prepare** :—chloroform 1; ox serum obtained as sterily as possible, 200 to 250. (2) Place in incubator 48 hr. with occasional shaking. (3) Test sterility. (4) **Prepare laked blood** :—D. W. 1; defibrinated ox blood obtained as sterily as possible 1. (5) **Prepare** :—chloroform 1; laked blood 200 to 250 c.c. (6) Place in incubator 48 hr. with occasional shaking. (7) Test sterility. (8) **Prepare with sterile precautions and without shaking, hæmoglobinized serum** :—chloroformed laked blood 1; chloroformed serum 20. (9) **Prepare with sterile precautions** :—hæmoglobinized serum at 45 C. 1; melted peptone agar (M4:11) 7. (10) Distribute with sterile precautions into T. T. (11) Slope. (12) Stack the T. T. in the horizontal position.<sup>2</sup> (13) Test sterility by incubation 48 hr.

**Notes.**—<sup>1</sup>For cultivation of meningococcus, etc. The medium should be clear and free from all trace of red colour. <sup>2</sup>To prevent unequal drying.

**M2:238.**<sup>1</sup>—(1) **Prepare with sterile precautions and without shaking hæmoglobinized serum** : chloroformed laked blood (M2:237) 1; chloroformed serum (M2:237) 50. (2) **Prepare with sterile precautions** :—hæmoglobinized serum at 45 C. 1; melted peptone agar (M4:11) at 45 C. 3. (3) Distribute with sterile precautions into T. T. (4) Slope. (5) Stack in the horizontal position.<sup>2</sup>

**Notes.**—<sup>1</sup>For cultivation of meningococcus from cerebro-spinal fluid. <sup>2</sup>To prevent the medium drying unevenly.

**M2:239.**—(1) **Prepare** :—12 per cent crystalline sod. carbonate 1; defibrinated blood 1. (2) **Prepare** :—alkaline defibrinated blood 3; 4 per cent melted nutrient agar 7.

**M2:24 LAKED BLOOD : M2:241.** (1) **Draw a rabbit's blood** from the carotid into 1·5 per cent sod. citrate. (2) Dilute with 0·85 S. S. S. to give a 5 per cent suspension of blood. (3) Add 10 per cent ether. (4) Shake to mix. (5) Leave the sediment 24 hr. (6) Draw off the laked blood into a sterile bottle. (7) **Add**<sup>1</sup> an excess of ether. (8) **Prepare** :—laked blood at 45 C. 1; melted tryptic agar (M4:711) 10 c.c.

**Note.**—<sup>1</sup>Add a few drops of ether to the blood each time the bottle is opened.

**M2·242.** (1) Collect blood at the slaughterhouse. (2) Keep in the ice chest 3 hr. (3) Remove the clot and mince finely. (4) Return the minced clot to the serum. (5) Add 2 vol. D. W. (6) Heat the mixture 20 min. at a temperature not exceeding 50 C. (7) Raise the temperature to boiling point. (8) Boil 10 min. (9) Pour the mixture on to a wet, thick, clean cloth. (10) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (11) Boil and skim off any coagulated protein which collects on the surface. (12) Add 0·5 c.c. glacial acetic acid per litre. (13) Boil 5 min. (14) Filter through well-wetted, thick, filter paper. (15) Add to the filtrate 1 per cent peptone and 0·5 per cent sod. chloride, (16) Steam 45 min. (17) Estimate and adjust the reaction of the medium (**S7·76, 7·8**). (18) Steam 30 min. (19) Filter, while hot, through well-wetted, thick, filter paper. (20) Distribute into T. T. (21) Sterilize (**S9·6**) 30 min. at 12 lbs.

**M2·243.**—(1) Prepare:—defibrinated ox blood at 70 C., 1; melted nutrient agar at 70 C., 20. (2) Steam 45 min. (3) Allow to solidify. (4) Steam to melt the medium. (5) Strain through glass wool, filled to a depth of  $\frac{1}{4}$  in. into a Buchner funnel, under slightly reduced pressure. (6) Distribute into T. T. (7) Sterilize (**S9·5**) at 100 C.

**M2·244.**—(1) Make a saturated sol. of defibrinated blood in 1 per cent saponin. (2) Add to melted nutrient agar.

**M2·245.**—(1) Boil 1 c.c. blood with 9 c.c. water. (2) Allow to deposit. (3) Add 0·5 c.c. clear colourless S. N. F. to 5 c.c. melted nutrient agar.

**Note.**—<sup>1</sup>For cultivation of *B. influenzae*, etc.

**M2·546.**<sup>1</sup> (1) Prepare: blood 1; N-1 sulphuric<sup>2</sup> acid 1; water 5. (2) Boil. (3) Store for use. (4) Make faintly alkaline to litmus at the time of use. (5) Prepare:—Blood fluid faintly alkaline to litmus at 45 C., 1; melted nutrient agar at 45 C., 5.

**Notes.**—<sup>1</sup>For cultivation of *B. influenzae*, etc. <sup>2</sup>The sulphuric acid sterilizes the blood. Brilliant green, which inhibits the growth of Gram positive bacteria, may be added.

**M2·247.**<sup>1</sup> (1) Lave defibrinated blood with the smallest possible amount of sterile D. W. (2) Add the laked blood to glucose agar (**M4·521**) in quantity sufficient to give a deep colour to the medium.

**Note.**—<sup>1</sup>For cultivation of meningococcus, etc.

**M2·25 CITRATED BLOOD : M2·251.**—(1) Add 1 c.c. citrated<sup>1</sup> blood to a T. T. of melted nutrient agar at 45 C. (2) Rotate the T. T. between the hands to distribute the blood through the agar.

**Note.**—40 c.c. blood to 1 c.c. sterile 10 per cent sod. citrate.

**M2·252.**—(1) Prepare : pigeon's blood 1 : 1 per cent citrated 0·85 S. S. S. 9. (2) Heat 30 min. at 65 to 70 C. (3) Filter through thick filter paper. (4) Prepare : filtrate at 45 C., 1 : melted nutrient agar faintly alkaline to litmus, at 45 C., 10.

**M2·253.**—(1) Bleed a rabbit directly from the carotid into 50 c.c. 5 per cent citrated 0·85 S. S. S. (2) Dilute the citrated blood with 0·85 S. S. S. to give a content of 5 per cent rabbit's blood. (3) Add ether to 10 per cent. (4) Shake to mix. (5) Allow to deposit 24 hr. (6) Distribute the supernatant, clear, laked blood in : stoppered bottles. (7) Add a little excess of ether to each bottle.<sup>1</sup> (8) Prepare : haemolyzed blood at 45 C., 2 : melted trypan-agar (**M4·7**) at 45 C., 100.

**Note.**—<sup>1</sup>Add a few drops ether also each time the bottle is opened.

**M2·254.**—(1) Prepare : citrated blood<sup>1</sup> at 45 C., 0·2 : melted nutrient agar at 45 C., 5. (2) Place in a water bath at 80 C. until the agar has become chocolate in colour. (3) Slope.

**Note.**—10 per cent citrated 0·85 S. S. S. 4 ; blood 10.

**M2·255.**<sup>1</sup> (1) Prepare : sterile fresh human blood 1 : 0·5 percent citrated 0·85 S. S. S., 4. (2) Heat in a water bath 30 min. at 61 to 68 C. (3) Filter through sterilized paper. (4) Prepare : blood fluid filtrate at 45 C., 1 : melted nutrient agar faintly alkaline to litmus at 45 C., 10.

**Note.**—<sup>1</sup>For cultivation of *B. influenzae*, etc.

**M2·26 OXALATED BLOOD : M2·261.**<sup>1</sup> (1) Collect 400 c.c. ox or sheep blood at the slaughterhouse in a sterile flask containing 30 c.c. 1 per cent ammon. oxalate and 0·5 c.c. formalin. (2) Mix well. (3) Allow to stand 30 min. (4) Dilute 1·4 with 0·85 S. S. S. (5) Leave 48 hr. (6) Prepare : oxalated blood 1 : nutrient medium at 45 C., 15.

**Note.**—<sup>1</sup>Used for cultivation of delicate organisms, such as gonococcus, meningococcus, pneumococcus.

**M2·27 DIGEST BLOOD : M2·271.** (1) Prepare : ox blood 500 : pepsin powder 15 : hydrochloric acid 15 : D. W. 1000. (2) Keep for 12 hr. at 40 to 42 C. (3) Boil 30 min. (4) Filter. (5) Make faintly alkaline to litmus. (6) Solidify with agar.

**M2·272.**<sup>1</sup> (1) Prepare with sterile precautions : quicklime and carbonate 0·25 : sterile 5 per cent trypsinized<sup>2</sup> 0·85 S. S. S. 60. (2) Distribute in quantities of 10 c.c. in sterile T. T. (3) Add to each T. T.

2 c.c. sterile human blood. (4) Incubate 8 d. or longer with occasional shaking. (5) Prepare with sterile precautions: blood digest at 45 C., 1; melted nutrient agar at 45 C., 9. (6) Distribute into T. T. or plates.

**Notes.**—<sup>1</sup>For cultivation of *B. influenzae*, <sup>2</sup>*e.g.*, Liq., trypticase Co. (Allen and Hanbury).

**M2'3 BLOOD SERUM OR PLASMA: M2'31 SERUM WATER: M2'311.** (1) Prepare: clear ox serum 1; water 3. (2) Steam 15 min. (3) Add litmus (**S7'783**) to give a deep blue tint, and 1 per cent sugar. (4) Sterilize (**S9'5**) 20 min. at 100 C., 3 d. or by filtration.

**M2'312.**—(1) Prepare:—serum 1; D. W. 3. (2) Sterilize 20 min. at 118 C. (3) Add sugar to 1 per cent. (4) Sterilize (**S9'5**) 20 min. 3 d. at 100 C.

**M2'32.**—Collect ox or sheep blood at the slaughterhouse in a sterile blood jar or in a bucket. (2) Place 24 hr. in cold storage. (3) Collect the serum. (4) Add 2 per cent. chloroform as preservative. (5) Prepare:—glucose bouillon (**M4'529**) 1; serum 3. (6) Coagulate (**M6'1**). (7) Sterilize (**S9'5**) 24 hr. after coagulation.

**M2'33.**—(1) Collect ox or sheep blood at the slaughterhouse<sup>1</sup> in a sterile blood jar or in a bucket. (2) Allow the blood to coagulate before removing it. (3) Separate the clot from the sides of the containing vessel with a sterile glass rod. (4) Place 24 hr. in a cool place. (5) Transfer the separated serum with a sterile pipette in quantities of 5 c.c. to sterile T. T. (6) Sterilize 30 min. at 58 C. on each of 8 successive days or 20 min. at 100 C 3 d. (7) Coagulate (**M6'1**). (8) Test sterility by incubation for 48 hr.

**Note.**—<sup>1</sup>Allow the first flow of blood to escape before collecting. If the blood be collected aseptically, sterilization is unnecessary.

**M2'34.**—(1) Prepare:—glycerin 5; ox serum 95. (2) Heat 30 min. at 56 C. in a water bath on each of two successive days. (3) Coagulate (**M6'1**) 24 hr. later. (4) Test sterility before use by incubation 48 hr.

**M2'35.**—(1) Prepare:—glucose bouillon (**M4'529**) 1; ox or sheep serum 3. (2) Coagulate (**M6'1**).

**M2'36.**—(1) Prepare:—10 per cent sod. hydroxide 1·5; ox serum 100. (2) Coagulate (**M6'1**).

**M2'37.**—(1) Prepare:—sterile or sterilized placental blood serum at 45 C., 1; melted nutrient agar 1 per cent acid to phenolphthalein at 45 C., 1.

**M2:38.**—(1) Coagulate (**M6:1**). (2) Add 0.85 S. S. S. to each T. T. slope to cover the medium. (3) Sterilize 60 min. at 115 C. (4) Pour off the salt sol.<sup>1</sup> at the time of use.

**Note.**—<sup>1</sup>The addition of salt sol. allows of satisfactory sterilization and keeps the medium moist till required.

**M2:39.**—(1) Prepare:—sterile human serum at 150 C., 1; melted nutrient agar at 45 C., 2.

**M2:4 BLOOD SERUM OR PLASMA: M2:41.** (1) Prepare and dissolve:—peptone 5; sod. chloride 2.5; glucose 2.5; agar 10; water 500. (2) Add calf serum 500. (3) Boil 20 min. (4) Filter while hot. (5) Distribute in T. T. (6) Sterilize 50 min. at 100 C. or 30 min. at 117 C.

**M2:42.**—(1) Sterilize pure glycerin. (2) Prepare with sterile precautions:—sterilized glycerin 7; sterile ox sterilized serum 100. (3) Coagulate (**M6:1**).

**M2:43.**<sup>1</sup>—(1) Prepare and dissolve:—agar 1.5; water 100. (2) Filter. (3) Distribute in T. T. (4) Sterilize at 120 C. (5) Cool to 40 C. (6) Add an equal vol. of sterile serum.<sup>2</sup> (7) Mix gently by rotating the tubes in the hands. (8) Slope. (9) Test sterility by incubation 48 hr.

**Notes.**—<sup>1</sup>The same medium as **M2:41** without the peptone, salt and glucose. <sup>2</sup>0.1 acetic fluid.

**M2:44.** (1) Mince finely fresh human placenta. (2) Add 500 gm. placenta and placental fluid to 100 c.c. water. (3) Prepare:—sod. chloride 5; glucose 10; nutrose 20; peptone 20; agar 25; placental fluid 1000. (5) Prepare:—melted placental fluid agar at 15 C., 3; ox serum at 45 C., 1. (6) Pour in plates.

**M2:45.**—(1) Prepare:—chloroform 2; blood serum 100. (2) Keep in a well-stoppered bottle in the dark 2 m. (3) Distribute in sterile T. T. with sterile precautions. (4) Coagulate<sup>1</sup> (**M6:1**).

**Note.**—<sup>1</sup>The chloroform will be driven off in the process.

**M2:46.**<sup>1</sup> (1) Prepare:—ox serum heated 30 min. at 56 C., 1; 0.85 S. S. S. or Locke's fluid,<sup>2</sup> 9.

**Notes.**—<sup>1</sup>For cultivation of *S. icterohæmorrhagæ*. <sup>2</sup>1. sod. chloride 9.2; sod. bicarbonate 0.05; pot. chloride 0.1; calc. chloride 0.1; 0.85 S. S. S., water 1000.

**M2:47.**<sup>1</sup>—(1) Prepare:—rabbit serum heated 30 min. at 56 C., 1; 0.85 S. S. S., 5.

**Note.**—<sup>1</sup>For cultivation of *S. icterohæmorrhagæ*.

**M2'48<sup>1</sup>.** (1) Prepare : sterile rabbit serum at 45 C. 15 ; Ringer's sol.<sup>2</sup> at 45 C. 15 ; melted nutrient agar at 45 C. 1. (2) Cover with a thin layer of paraffin oil.

**Note.**—<sup>1</sup>For cultivation of *S. tetradymorpha*, which is an obligatory aerobe.  
<sup>2</sup>Sod. chloride 0.88 ; pot. chloride 0.025 ; calc. chloride 0.02 ; sod. bicarbonate 0.015 ; D. W. 100.

**M2'49.<sup>1</sup>** (1) Prepare : sterile rabbit serum<sup>2</sup> heated 30 min. at 58 to 60 C. and covered with a layer of sterile paraffin oil.

**Notes.**—<sup>1</sup>For cultivation of spirochaetes. <sup>2</sup>Diluted or undiluted.

**M2'5 BLOOD SERUM OR PLASMA. M2'51<sup>1</sup>.** (1) Fill the serum into tall T. T. (2) Heat in the upright position at 65 C. (3) Remove as soon as the serum begins<sup>2</sup> to set.

**Notes.**—<sup>1</sup>For cultivation of spirochaetes. <sup>2</sup>The heat retained in the tube will complete the coagulation. A soft, almost transparent, coagulum is formed.

**M2'52.<sup>1</sup>**—(1) Prepare :—unheated clear sterile horse serum 1 ; glucose bouillon (**M4'529**), 0.6 per cent. acid to phenolphthalein, 20.

**Note.**—<sup>1</sup>For cultivation of meningococcus.

**M2'53.** (1) Collect ox or sheep blood at the slaughterhouse in a sterile blood jar. (2) Allow the blood to coagulate. (3) Detach the clot. (4) Place in the ice chest. (5) Pipette off the serum with a sterile pipette. (6) Keep the serum in the ice chest till required.<sup>1</sup> (7) Distribute a portion of the serum in quantities of 5 c.c.<sup>2</sup> in T. T. (8) Sterilize 2 d. 30 min. at 60 C. (9) Coagulate (**M6'1**).

**Notes.**—<sup>1</sup>It is not advisable to coagulate more than is actually required at the time.  
<sup>2</sup>The small quantity 5 c.c. is quickly coagulated.

**M2'54<sup>1</sup>.**—(1) Collect ox or sheep blood at the slaughterhouse in a sterile blood jar. (2) Leave the jar at the slaughterhouse to avoid the shaking up consequent on transportation. (3) Transfer the separated serum 24 hr. later with a sterile pipette to a sterile flask. (4) Transport to laboratory. (5) Add ether to 5 per cent. (6) Cork tightly. (7) Leave 24 hr. (8) Replace the cork with a sterile wool plug. (9) Keep the flask and contents in a water bath<sup>2</sup> for 3 hr. at 50 C. to drive off the ether. (10) Prepare :—serum at 45 C. 25 ; melted nutrient agar 100.

**Notes.**—<sup>1</sup>For cultivation of meningococcus. <sup>2</sup>The flask may be placed overnight in the incubator instead.

**M2'55<sup>1</sup>.**—(1) Prepare pyrogalllic acid sol. :—pyrogalllic acid 1 ; sod. hydroxide 2 ; D. W. 100. (2) Keep several weeks before use. (3) prepare :—serum 10 ; pyrogalllic acid sol. 1. (4) Coagulate (**M6'1**). (5) Sow by passing a pipette containing culture material between the

medium and the wall of the T. T. (6) Seal the T. T. hermetically. (7) Incubate.

**Notes.**—<sup>1</sup>For cultivation of spirochaetes.

**M2·56.**<sup>1</sup> (1) Prepare :—serum 1 : 1-300 gentian violet in 0·85 S.S.S. (2) Coagulate (**M6·1**).

**Notes.**—<sup>1</sup>For cultivation of spirochaetes.

**M2·57.** (1) Add 1 c.c. formalin to 500 c.c. horse serum. (2) Add 1 per cent ammonia to neutralize to litmus. (3) Prepare :—formalinized serum 1 : D. W. 2. (4) Sterilize (**S9·6**) 15 min. at 110C. (5) Prepare :—sterilized formalinized serum 1 : pepsin digest agar (**M3·921**) 3.

**Notes.**—<sup>1</sup>For primary cultivation of meningococcus.

**M2·58.**—(1) Prepare :—horse serum 3 : D. W. 1. (2) Distribute in quantity to nearly fill T. T. (3) Close the T. T. with a rubber cork. (4) Heat 1 hr. at 60C. in a water bath. (5) Heat, 24 hr. later 1 hr. at 70C. in a water bath. (6) Heat, 24 hr. later at 70C. until the medium becomes syrupy. (7) Keep in the ice chest till required for use.

**Notes.**—<sup>1</sup>For cultivation of spirochaetes.

**M2·59.**<sup>1</sup>—(1) Prepare :—30 per cent glucose 10 : 1-100 sulphuric acid 3 : horse serum 100. (2) Tinge with sterile litmus sol. (**S7·783**). (3) Pour into Petri dishes. (4) Keep 75 min. at 75 to 78C. (5) Decant condensation water. (6) Dry the plates in the incubator.

**Notes.**—<sup>1</sup>For cultivation of *B. diphtheriæ*.

**M2·6 BLOOD SERUM OR PLASMA : M2·61.** (1) Prepare :—horse serum 1 : D. W. 3. (2) Add 0·5 c.c. 10 per cent sod. hydroxide<sup>1</sup> per 100 c.c. serum fluid. (3) Sterilize (**S9·6**) in autoclave at 112C. (4) Prepare<sup>2</sup> :—sterilized serum fluid at 80C.<sup>3</sup> 1 : melted pepsin digest agar (**M3·9211**) at 80C., 2. (5) Slope.

**Notes.**—<sup>1</sup>The addition of alkali permits of sterilization without coagulation. <sup>2</sup>The serum fluid may be rendered only just alkaline to litmus by the addition of hydrochloric acid before addition to the agar—as for example, when required for the cultivation of *B. diphtheriæ*, etc. <sup>3</sup>In order to avoid precipitation of colloids.

**M2·62.**<sup>1</sup>—(1) Prepare :—Sheep serum 3 : glucose 0·5 per cent : pot. sulphocyanide 1 per cent : 0·5 per cent neutral red 2 per cent : bouillon 1.

**Notes.**—<sup>1</sup>For cultivation of *B. diphtheriæ*, etc.

**M2·63.**<sup>1</sup>—(1) Prepare :—Serum 1 (T. W. 2. (2) Boil the serum turns milky. (3) Prepare :—Milky serum fluid 100 : phosphate extract

(T5·1) 15. (4) Digest in water bath 24 hr. at 60C. (5) Filter through paper. (6) Distribute in T. T. (7) Sterilize (S9·6) 15 min. at 120C.

**Notes.**—<sup>1</sup>For indol test.

**M2·64.**<sup>1</sup>—(1) Prepare :—Finely minced ox heart 1; D. W. 1. (2) Heat the mixture 20 min. at a temperature not exceeding 50C., with constant stirring. (3) Raise the temperature to boiling point. (4) Boil 10 min. (5) Pour the mixture on to a wet, thick, clean cloth. (6) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (7) Add peptone 1 per cent; di-sod. phosphate 1 per cent; prepared agar (M1·11) 3 per cent. (8) Steam gently 2½ hr. to dissolve the agar. (9) Allow to cool to 60C. (10) Clarify with white of egg (M5·1). (11) Bring the vol. to its original amount. (12) Estimate and make the reaction 0·6 per cent acid to phenolphthalein. (13) Add to the melted nutrient agar, 2·5 per cent glucose. (14) Steam 30 min.<sup>2</sup> (15) Distribute in quantities of 4 c.c. into T. T. (16) Keep till required for use in the ice chest. (17) Prepare sterile 10 c.c. centrifuge tubes each containing 2 c.c. sterile 2 per cent sod. citrate. (18) Have in readiness corks or rubber bungs, contained in alc., to fit the centrifuge tubes. (19) Fill up the centrifuge tubes with human blood sterilely aspirated. (20) Replace the wool plugs of the centrifuge tubes by corks after burning off the alc. (21) Centrifuge. (22) Prepare with sterile precautions :—Centrifuged blood fluid at 45C., 75; melted nutrient agar contained in the T. T. at 45C., 4. (23) Roll the T. T. between the hands to mix. (24) Test sterility by incubating 48 hr.

**Notes.**—<sup>1</sup>For cultivation of gonococcus. <sup>2</sup>It is better to rely on one steaming for sterilization than to sterilize by steaming on three successive days.

**M2·65.**<sup>1</sup>—(1) Pour into a Petri dish 2 per cent simple watery sol. of agar. (2) Allow to solidify (3) Pour on to the surface of the agar 5 c.c. serum bouillon (M2·65). (4) Coagulate (M6·1).

**Notes.**—<sup>1</sup>A method of economising serum medium for cultivation of *B. diphtheriæ*.

**M2·66.**—(1) Prepare :—Human, ox, ass, mule or horse serum 2; bouillon 1. (2) Sterilize (S9·3) 60 min. at 57C. on two successive days.

**M2·67.**<sup>1</sup>—(1) Prepare :—Peptone 10; sod. chloride 9; calc. chloride 0·25; pot. chloride 0·42; glucose 25; meat extract (M3·61) 1000, of reaction 0·6 per cent acid to phenolphthalein. (2) Add 1 c.c. citrated human plasma (M2·63) to each 5 c.c. medium.

**Notes.**—<sup>1</sup>For cultivation of gonococcus.

**M2·68.**—(1) Prepare : Chloroform 3 ; serum 100. (2) Shake to mix. (3) Leave 3 d. with occasional shaking. (4) Prepare : Chloroformed serum 3 ; glucose bouillon (**M4·529**) 1. (5) Distribute into Petri dishes. (6) Heat 6 hr. at 75°C. (7) Raise the temperature, 24 hr. later, slowly to 80°C. and keep 1 hr. at this temperature. (8) Sterilize (**S9·5**) 30 min. at 100°C.

### **M2·7 BODY FLUID.<sup>1</sup>**

**Notes.**—<sup>1</sup>Other than blood and blood serum.

**M2·71 BODY FLUID : M2·711.**—(1) Prepare : Body fluid<sup>1</sup> 1 ; pure neutral glycerin 1. (2) Leave till sterile on culture test. (3) Prepare : Glycerinated body fluid 0·6 ; nutrient agar 100.

**Notes.**<sup>1</sup>—Acetic fluid, pleuritic fluid, hydrocele fluid, ovarian fluid, milk, urine, etc.

**M2·712.**—(1) Prepare : Glycerin 20 ; 10 per cent sod. hydroxide 20 ; agar 20 ; body fluid 1000.

**M2·72 ACETIC FLUID : M2·721.**—(1) Prepare : Acetic fluid 1 ; bouillon<sup>1</sup> 2. (2) Sterilize (**S9·3**) in the water bath 30 min. at 56°C. on five successive days. (3) Test sterility before use by incubation 48 hr.

**Notes.**—<sup>1</sup>Or melted nutrient agar.

**M2·722.**—(1) Collect acetic fluid with sterile precautions. (2) Leave overnight to allow of separation of clot. (3) Add 50 c.c. N-1 sod. hydroxide per litre. (4) Steam 20 min. (5) Add 1 gm. agar per litre after making it into a paste or suspension with a little of the alkaline acetic fluid. (6) Steam to dissolve the agar. (7) Filter, while hot, through thick, filter paper by placing filter funnel, stand, and receptacle for filtrate in the sterilizer. (8) Dissolve 10 gm. glucose<sup>1</sup> per litre in the hot, filtered, nutrient agar. (9) Distribute into T. T. (10) Sterilize (**S9·5**).

**Notes.**—<sup>1</sup>Add glycerin 50 c.c. per litre also if desired.

**M2·723.**—(1) Prepare :—Nutrose 1 ; acetic fluid 15 ; D. W. 35. (2) Raise slowly to boiling temperature with frequent shaking to prevent burning. (3) Boil or steam 30 min. (4) Clarify (**M5·1**) and filter through thick, filter paper. (5) Prepare :—Filtrate at 45°C. 1 ; melted nutrient agar at 45°C. 2. (6) Steam 30 min. (7) Distribute into T. T. (8) Sterilize.

**M2·724.**<sup>1</sup>—(1) Prepare :—Acetic fluid at 45°C. 1 ; nutrient glucose bouillon (**M4·529**) faintly alkaline to litmus at 45°C. 3.

**Notes.**—<sup>1</sup>For cultivation of meningococcus.

**M2·725.**—(1) Prepare :—Acetic fluid 10 ; sterile defibrinated sheep blood 20 ; 1 grm. maltose dissolved in bouillon 3 ; nutrient agar 60.

**M2·726.**<sup>1</sup>—(1) Prepare :—Sterile acetic fluid at 45C., 7 ; sterile standard litmus sol. (**S7·787**) at 45C. 3 ; sterile 10 per cent sugar at 45C., 2 ; sterile N-1 sod. hydroxide at 45C., 0·15 ; melted nutrient agar at 45C., 21.

**Notes.**—<sup>1</sup>For cultivation of meningococcus.

**M2·727.**—(1) Prepare :—10 per cent sod. hydroxide<sup>1</sup> 0·5 ; acetic fluid 10. (2) Sterilize (**S9·6**) in autoclave at 112C. (3) Allow to cool. (4) Make faintly alkaline to litmus with 1-15 hydrochloric acid. (5) Sterilize (**S9·6**) in autoclave at 112C. (6) Prepare : Sterilized acetic fluid at 80C.,<sup>2</sup> 1 ; melted pepsin digest agar (**M3·921**) at 80C., 2.

**Notes.**—<sup>1</sup>The addition of the alkali permits of sterilization without coagulation. <sup>2</sup>In order to avoid precipitation of colloids.

**M2·728.**—(1) Prepare :—Acetic fluid 1 ; bouillon 2.

**M2·73.**—**URINE : M2·731.** (1) Distribute into T. T. freshly passed urine diluted to S. G. 1010. (2) Sterilize (**S9·5, 9·6**).

**M2·732.**—(1) Collect urine fresh. (2) Boil. (3) Filter. (4) Sterilize (**S9·5, 9·6**).

**M2·74.**—**PANCREATIC FLUID : M2·741.**—(1) Prepare :—Pancreatin 2 ; sod. chloride 5 ; water 100. (2) Sterilize (**S9·7**) by filtration through a porcelain candle.

**M2·75 BILE.**—*vide* **M3·31.**

**M2·8 EGG : M2·81 WHOLE EGG : M2·811.**—(1) Clean thoroughly the eggs to be used. (2) Wash with 5 per cent carbolic acid. (3) Allow to dry partially. (4) Heat the ends of the eggs gently in the flame to dry them. (5) Pierce, with sterile precautions, both ends. (6) Blow<sup>1</sup> the whole contents of the eggs into a sterile, wide-mouthed, glass stoppered bottle containing glass beads. (7) Add<sup>2</sup> 1 vol. 0·85 S. S. S. for each 3 vol. egg contents. (8) Shake well to mix. (9) Strain<sup>3</sup> through cloth. (10) Distribute into T. T. (11) Coagulate (**M6·11**). (12) Fill into each T. T. 0·85 S. S. S. to cover the medium. (13) Sterilize<sup>4</sup> (**S9·6**) in autoclave. (14) Pour off the salt sol. at the time of use.

**Notes.**—<sup>1</sup>The eggs may be carefully broken and the contents allowed to fall into the bottle. <sup>2</sup>If desired 3·75 c.c. N-1 sod. hydroxide per 100 c.c. egg mixture may be added and also 1 per cent glucose at this stage. <sup>3</sup>Add at this stage, if desired, some drops of alc. basic fuchsin to give a slight pink colour to the medium. <sup>4</sup>It is very usual to dispense with the addition of salt sol. and subsequent sterilization. In that case sterility should be tested before use by incubation for 48 hr.

**M2'812.**—(1) Make into a suspension the whole contents of one egg with 12 c.c. glucose bouillon (**M4'529**). (2) Place the suspension in T. T. (3) Coagulate (**M6'1**). (4) Sterilize (**S9'5**) 20 min. at 100°C. 3 *d*. 24 hr. after completion of coagulation. (5) Test sterility before use.<sup>1</sup>

**Notes.**—<sup>1</sup>Use for anaerobic culture and for isolation of *B. diphtheriae*. Add 1 c.c. glycerin to each T. T. before final sterilization to convert into a medium suitable for cultivation of *B. tuberculosis*. The addition of a few drops of 1 per cent neutral red gives a colour to the medium which serves to show up colonies, and serves as an indicator of the production of acid.

**M2'813.**<sup>1</sup>—(1) Add the contents of one egg to 300 c.c. water. (2) Shake to mix. (3) Raise the temperature slowly to boiling point with frequent shaking. (4) Distribute into T. T. (5) Sterilize (**S9'5**).

**Notes.**—<sup>1</sup>For cultivation anaerobically of wound organisms.

**M2'814.**—(1) Place whole eggs in 10N sod.<sup>1</sup> hydroxide for 10 *d*. Remove the shells. (3) Cut the eggs into fine slices. (4) Wash for 2 hr. in running water. (5) Steam 60 min. (6) Transfer each slice of egg by means of sterile forceps to a sterile Petri dish. (8) Sterilize 20 min. at 100°C. 3 *d*.

**Notes.**—<sup>1</sup>The egg white is solidified.

**M2'815.**—(1) Prepare :—6 per cent glycerinated 0.85 S. S. S. 1 ; beaten up egg 3. (2) Coagulate (**M6'1**).

**M2'816.**—(1) Use a thoroughly fresh egg. (2) Shake vigorously to mix the contents. (3) Wash the shell in 1:4000 mercury bichloride. (4) Dry with sterile filter paper. (5) Flame the narrow end until the shell blackens. (6) Make a hole in this end with a sterile metal point. (7) Sow through the hole with a platinum needle or a pipette. (8) Close the whole with melted sealing wax. (9) Coat the egg with a layer of collodion.

**M2'817.**—(1) Boil an egg hard. (2) Remove the shell. (3) Cut up the egg into pieces. (4) Place in Petri dishes. (5) Sterilize at 115°C.

**M2'818.**—(1) Beat up the contents of several fresh eggs. (2) Prepare :—Beaten up egg 3 ; horse heart trypan digest (**M4'713**) 1. (3) Filter through muslin. (4) Distribute into T. T. (5) Coagulate (**M6'1**).

**M2'819.**—(1) Mince finely beef or veal. (2) Add 100 c.c. water and 15 per cent glycerin. (3) Leave to extract 24 hr. (4) Collect all the fluid including that obtained by squeezing the meat-extracted meat juice. (5) Sterilize the outer surface of eggs by placing them for 10 min. in 70 per cent alc. or by pouring boiling water over them. (6) Allow

the eggs into a sterile beaker and mix. (7) Filter the egg mixture through sterile gauze. (8) Prepare glycerinated meat juice 1; 1 per cent alc. gentian violet 0.03; egg mixture 2. (9) Distribute into T. T. (10) Coagulate (**M6.1**). (11) Heat, 24 hr. after coagulation for 60 min. at 75C. on two successive days.

**M2.82—WHOLE EGG : M2.821.**—(1) Prepare egg stock sol.:—White of 2 eggs; yolk of one egg; N-1 sod. hydroxide 6 c.c. (2) Mix. (3) Add water 500 c.c. (4) Heat very slowly to 90C. (5) Distribute into flasks. (6) Sterilize in the autoclave. (7) Prepare at the time of use, with sterile precautions:—egg stock sol. at R. T. 1; bouillon at R. T. 1, or egg stock sol. at 45C., 1; melted nutrient agar at 45C., 10.

**M2.822.**<sup>1</sup>—(1) Prepare<sup>2</sup> egg stock sol.:—White of 2 eggs; yolk of one egg; N-1 sod. hydroxide 6 c.c. (2) Mix. (3) Add water 500 c.c. (4) Heat very slowly to 95C. (5) Keep at 95C. 60 min. (6) Filter through cotton wool. (7) Distribute into flasks. (8) Sterilize in autoclave. (9) Prepare at the time of use with sterile precautions:—Egg stock sol. at R. T. 1; bouillon at R. T. 5, or egg stock sol. at 45C., 1; melted nutrient agar at 45C., 10.

**Notes.**—<sup>1</sup>Alkaline egg medium. <sup>2</sup>A clearer medium is obtained by using white of 2 eggs; N-1 sod. hydroxide 4; water 330.

**M2.823.**<sup>1</sup>—(1) Mix the contents of one egg with an equal quantity of water. (2) Add to the mixture an equal amount. of 6.5 per cent anhydrous sod. carbonate. (3) Sterilize (**S9.5**) 1 hr. at 100C. (4) Keep<sup>2</sup> as stock. (5) Add 1 vol. of the stock mixture to 9 vol. ordinary peptone water (**M4.12**). (6) Distribute into T. T. or flasks. (7) Sterilize (**S9.5**).

**Notes.**—<sup>1</sup>A medium equally, or more, selective for *V. cholerae* than peptone water. Will keep several weeks.

**M2.824.**<sup>1</sup>—(1) Proceed as in steps (1) to (4) of **M2.823**. (2) Add 1 vol. of the stock mixture to 5 vol. neutral 1 per cent cane sugar agar. (3) Add to the mixture while melted 1 per cent freshly prepared 0.5 per cent neutral red. (4) Slope or pour on plates.

**Notes.**—<sup>1</sup>For the isolation of *V. cholerae*. The medium has an orange tint and the colonies of *V. cholerae* have a deep red centre.

**M2.83 WHITE OF EGG : M2.831.**—(1) Proceed as in steps (1) to (6) of **M2.816** (2) Aspirate the white of the egg into a sterile pipette. (3) Distribute into T. T. (4) Coagulate (**M6.1**).

**M2.84 YOLK OF EGG : M2.841.**—(1) Wash\* the eggs with hot soap and water. (2) Dip into alc. (3) Allow the alc. to drain off. (4) Set fire to the alc. wetting the eggs. (5) Make a hole in the shell with

a sterile metal instrument. (6) Allow the white to run off. (7) Mix together the yolk of 6 eggs. (8) Add 100 c.c. bouillon neutral to litmus and containing 1 per cent glucose<sup>1</sup> or 5 per cent glycerin.<sup>2</sup> (9) Shake to mix. (10) Distribute into T. T. (11) Coagulate (M6'1).

**Notes.**—<sup>1</sup>For cultivation of *B. diphtheriae*. <sup>2</sup>For cultivation *B. tuberculosis*.

**M2'9 FATS, FATTY ACIDS, SALTS OF FATTY ACIDS, OIL:**  
**M2'91 OIL: M2'911<sup>1</sup> SPERM OIL.**—(1) Add 5 per cent sperm oil to beaten up and filtered egg medium (M2'811). (2) Add glycerinated 0.85 S. S. S. to give 5 per cent of glycerin. (3) Shake the mixture in a bowl of hot water with a Bunsen burner underneath, to melt the wax. (4) Distribute into T. T. and keep in a bowl of hot water to prevent the wax separating out. (5) Shake to mix. (6) Place low down in the steamer and steam 4 min. to coagulate the egg. (7) Sterilize by placing high up in the steamer for 1 hr. on three successive days.

**Notes.**—<sup>1</sup>For cultivation of *B. tuberculosis*.

**M2'912 OLIVE OIL.**—(1) Proceed as for sperm oil (M2'911).

**M2'92 OLEIC ACID: M2'921.<sup>1</sup>**—(1) Prepare: Glycerin 2: oleic acid 0.1: melted nutrient agar neutral to phenolphthalein 100.

**Notes.**—<sup>1</sup>For cultivation of *B. acnes*.

**M2'922.<sup>1</sup>**—(1) Prepare: Sterile oleic acid 2: sterile acetic fluid 20: sterile 1 per cent neutral red 0.8: sterile nutrient agar 100. (2) Test sterility.

**Notes.**—<sup>1</sup>For cultivation of *B. acnes*.

**M2'923.**—(1) Prepare: Oleic acid 1: glycerin 2: melted nutrient agar 100.

**M2'93 OLEATE: M2'931.<sup>1</sup>**—(1) Prepare: Sterile 2 per cent neutral sod. oleate at 45C., 5: sterile washed human erythrocytes at 45C., 1: melted nutrient agar 0.4 per cent acid to phenolphthalein, at 45C., 95.

**Notes.**—<sup>1</sup>For cultivation of *B. influenzae*.

**3 MEDIA PREPARATION AND COMPOSITION: M3'1 GLYCERIN: M3'11 BOUILLON, PEPTONE. AGAR: M3'111.**—(1) Prepare: Glycerin 5: bouillon 100.

**Notes.**—<sup>1</sup>Convert into glycerin agar if required.

**M3'112.**—(1) Prepare:—Glycerin 10; N-1 sod. hydroxide 3; nutrient agar<sup>1</sup> 87.

**Notes.**—<sup>1</sup>For cultivation of *V. cholerae*. <sup>2</sup>May be replaced by 2 per cent peptone water (M4'2).

**M3·2 INDICATOR.<sup>1</sup>**

**Notes.**—<sup>1</sup>*v.* also sugar media (M4·5) and inhibitory substance (M3·3) media.

**M3·21 LITMUS: M3·211.** (1) Add sufficient sterile litmus sol. (S7·783) to tint the medium a dark lavender colour.

**M3·212.**—(1) Add sat. litmus sol. (S7·786) to give<sup>1</sup> a distinct blue colour.

**Notes.**—<sup>1</sup>The litmus sol. must not be so alkaline as to alter appreciably the reaction of the medium.

**M3·22 ROSOLIC ACID.<sup>1</sup>**

**Notes.**—<sup>1</sup>Coralline.

**M3·221.**—(1) Prepare:—Rosolic acid indicator sol.<sup>1</sup> 2; peptone water (M4·12) 100. (2) Steam 30 min. (3) Filter through thick filter paper. (4) Distribute in T. T. (5) Sterilize.

**Notes.**—<sup>1</sup>0·5 per cent rosolic acid in 80 per cent alc.

**M3·23 CHINA BLUE: M3·231 CHINA BLUE-ROSOLIC ACID.—**

(1) Prepare:—China blue 0·025; rosolic acid or its sodium salt 0·05; nutrient medium 1000.

**M3·232.<sup>1</sup>**—(1) Prepare:—Peptone 5 gm.; di-pot. phosphate 1 gm.; agar 15 gm. D. W. 1000 c.c. (2) Add to each 100 c.c. melted medium before using:—20 per cent lactose 5 c.c.; 5 per cent glucose 1 c.c.; 1 per cent alc.<sup>2</sup> rosolic acid 1 c.c.; 0·5 per cent china blue 1 c.c.

**Notes.**—<sup>1</sup>For isolation of *B. dysenteriae*. The p H value of this medium, which requires no adjustment and does not need to be filtered when used on plates, is 7·4 to 7·5. <sup>2</sup>90 per cent alc.

**M3·233 CHINA BLUE PHENOL RED.<sup>1</sup>**—(1) Prepare: China blue 0·025; phenol red 0·01; nutrient medium 1000.

**Notes.**—<sup>1</sup>Phenol sulphone phthalein.

**M3·24 BROM CRESOL PURPLE: M3·241.** (1) Prepare:—Brom cresol purple indicator sol.<sup>1</sup> (S7·779) 1; nutrient medium 1000.

**Notes.**—<sup>1</sup>1·6 per cent brom cresol purple in 95 per cent alc.

**M3·242** (1) Prepare: Brom cresol purple indicator sol.<sup>1</sup> 4; nutrient medium 100.

**Notes.**—<sup>1</sup>To prepare. Dissolve the brom cresol purple in a minimum amount of alc. Make up to 9·01 per cent with water. The colour given to the medium disappears at pH = 5 which is considerably below the curdling point of milk.

**M3·243.**—(1) Add brom cresol purple indicator sol. (S7·779) to milk to give the medium a distinct colour.

**M3·244 BROM CRESOL PURPLE-CRESOL RED.** (1)

Prepare: Brom cresol purple-cresol red indicator sol.<sup>1</sup> 1; nutrient medium 1000.

**Notes.**—<sup>1</sup>1·6 per cent brom cresol purple in 95 per cent alc. 0·5; 1·6 per cent cresol red in 95 per cent alc. 0·5. This compound indicator gives a wide range pH = 8 to pH = 5.

**M3·25 CRESOL RED: M3·251.**—(1) Prepare:—Cresol red indicator sol.<sup>1</sup> 1; nutrient medium 1000.

**Notes.**—<sup>1</sup>1·6 per cent cresol red in 95 per cent alc.

**M3·26 NEUTRAL RED: M3·261.**—(1) Prepare:—Sugar 5; 1 per cent neutral red 5; nutrient medium 1000.

**M3·27 CALC. CARBONATE.**—(1) Add to melted neutral glucose<sup>1</sup> agar sufficient sterilized precipitated chalk to render the medium white and opaque.<sup>2</sup>

**Notes.**—<sup>1</sup>Or other sugar. <sup>2</sup>Organisms which ferment the sugar produce acids and make the medium clear.

**M3·3 INHIBITORY SUBSTANCE.<sup>1</sup>**

**Notes.**—<sup>1</sup>Most of these *inhibit* the growth of organisms but differentially, and thus the media are known as "selective" media.

**M3·31 BILE AND BILE SALTS.<sup>1</sup>**

**Notes.**—<sup>1</sup>For differentiation typhoid group organisms before further sowing.

**M3·311.**—(1) Procure ligatured gall bladders (ox) from the slaughterhouse. (2) Puncture the bottom of the gall bladder with a knife. (3) Collect the bile. (4) Reject any bile which shows turbidity. (5) Distribute in T. T. or small bottles<sup>1</sup> in quantities of 5 to 10 c.c. (6) Sterilize in autoclave.

**Notes.**—<sup>1</sup>Rubber caps may be used to replace the cotton wool plug in these bottles and the medium may then be sent out for inoculation with test blood.

**M3·312.**—(1) Procure clear bile from the slaughterhouse. (2) Add 1 gm. peptone and 1 gm. glucose per 100 c.c. bile. (3) Steam 15 min. (4) Filter, while hot, through well-wetted, thick, filter paper. (5) Distribute into T. T. (6) Sterilize in autoclave.

**M3·313.**—(1) Prepare:—Peptone 1; glycerol 1; agar 10.

**M3·314.**—(1) Prepare:—Sod. taurocholate 10; glucose 10; peptone 40; litmus sol. to colour the medium a deep purple sol. (D. W. 1000.

**M3·315.**—(1) Prepare:—Sod. taurocholate 5; essential oil 1; ammon. citrate 0·5; peptone 10; agar 15; D. W. 1000.

**M3·316.**—(1) Prepare:—Peptone 20 gm.; sod. taurocholate 2 gm.; T. W. 1000 c.c. and make faintly alkaline to litmus. (2) Steam 40 min. (3) Add 15 gm. powdered agar, making it into a paste at temperature

before addition, with a little of the taurocholate peptone sol. (4) Steam gently  $2\frac{1}{2}$  hr. to bring the agar thoroughly into solution. (5) Bring the volume up to 1000 c.c. by the addition of water. (6) Cool to  $60^{\circ}\text{C}$ ., clarify and filter (**M5·1**). (7) Dissolve 10 gm. lactose<sup>1</sup> in 15 c.c. sterile water and steam 15 min. (8) Add the lactose sol. to the hot, clear nutrient agar. (9) Add 5 c.c. freshly prepared sterile 1 per cent. neutral red<sup>2</sup> by means of a sterile pipette. (10) Distribute the resulting agar, which is deep red, into flasks or T. T. (11) Steam 25 min.

**Notes.**—<sup>1</sup>Or other sugar if desired. <sup>2</sup>Litmus sol. may be used, especially in the case of the fluid medium.

**M 3·317.**—(1) Prepare : Peptone 1 ; glycerin 1 ; sod. taurocholate 2·5 ; water 100.

**M3·32 CARBOLIC ACID : M3·321.**—(1) Prepare :—Strong hydrochloric acid 4 ; 5 per cent carbolic acid 100. (2) Leave 3 d. (3) Add with a sterile pipette in quantities of 0·1, 0·2, and 0·3 c.c. to T. T. containing 10 c.c. sterile bouillon. (4) Test sterility before use by incubation 48 hr.

**M3·322.**—(1) Prepare :—Pure carbolic acid 1 to 5 ; bouillon 1000. (2) Sterilize.

**M3·323.**—(1) Prepare : Peptone 30 ; agar 20 ; 5 per cent carbolic acid<sup>1</sup> 0·05 ; water 1000, made faintly alkaline to litmus.

**Notes.**—<sup>1</sup>Add to the sterilized and melted agar medium just before use.

**M3·324.**—(1) Prepare :—Peptone 30 ; lactose 20 ; agar 20 ; sterile litmus sol.<sup>1</sup> (**S7·723**) 100 ; 5 per cent carbolic acid<sup>1</sup> 0·05 ; water 1000 made faintly alkaline to litmus.

**Notes.**—<sup>1</sup>Add to the sterilized and melted sugar agar medium just before use.

**M3·33 CRYSTAL VIOLET : M3·331.**—(1) Mince finely fat-free beef. (2) Add 1500 gm. to 2000 c.c. water. (3) Heat the mixture 20 min. at a temperature not exceeding  $50^{\circ}\text{C}$ . over a free flame. (4) Skim off fat floating on the surface. (5) Raise the temperature to boiling point. (6) Boil 10 min. (7) Pour the meat and fluid on to a wet, thick, clean cloth. (7) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (8) Filter the fluid collected through thick, well-wetted, filter paper. (9) Add to the filtrate, peptone 20 gm. ; nutrose 20 gm. ; sod. chloride 10 gm. and see that the mixture is alkaline to litmus. (10) Steam 45 min. (11) Add 60 gm. prepared fibre agar (**M1·11**). (12) Steam gently  $2\frac{1}{2}$  hr. or heat 15 min. at  $118^{\circ}\text{C}$ . to bring the agar thoroughly into sol. (13) Make faintly alkaline to litmus. (11) Filter, while hot, through well-wetted, thick,

filter paper by placing filter funnel, stand, and receptacle for filtrate in the steam sterilizer and steaming until filtration is completed. (15) Boil 300 c.c. litmus sol. (S7-783) 10 min. and dissolve in it 30 grm. chemically pure lactose. (16) Boil the lactose litmus sol. 15 min. (17) Mix hot lactose litmus sol. and the melted nutrose agar. (18) Make faintly alkaline,<sup>1</sup> if necessary, by the addition of 10 per cent sod. hydroxide. (19) Add 4 c.c. hot 10 per cent sod. hydroxide and 20 c.c. hot sterile 0·1 per cent chemically pure crystal violet. (20) Distribute in flasks. (21) Pour large plates for use, with a layer of medium about 4 mm. thick. (22) Place the plates so made, in the inverted position<sup>2</sup> in the incubator without their covers and resting on a support.

**Notes.**—<sup>1</sup>Until the red foam developed in shaking becomes violet blue. <sup>2</sup>To allow the surface of the medium to dry. The support used may be the Petri plate cover.

### M3-34 BRILLIANT GREEN.<sup>1</sup>

**Notes.**—<sup>1</sup>Brilliant green sulphate crystals, zinc-free.

**M3-341.**—(1) Prepare:—Sod. taurocholate 5; peptone 30; sod. chloride 5; lactose 10; powdered agar 30; 1-1000 brilliant green 20; 1 per cent picric acid 20; water 1000 of reaction 1·5 per cent acid to phenolphthalein.

**M3-342.**—(1) Prepare:—0·1 per cent brilliant green 10; 1 per cent picric acid 10; lemco nutrient agar 1500.

**M3-343.**<sup>1</sup>—(1) Prepare:—Peptone water (M4-12) 5; 1-10000 brilliant green 0·25.

**Notes.**—<sup>1</sup>Used as a selective medium for the typhoid group before further sowing.

**M3-344.**—(1) Add 0·1, 0·2, 0·35, 0·5 and 0·7 c.c. 1-10000 freshly prepared brilliant green to a series of T. T. containing 10 c.c. peptone water of reaction pH 7·2. (2) Add also 0·2 c.c. 1-1000 telluric acid<sup>1</sup> to each T. T.

**Notes.**—<sup>1</sup>To suppress mesite fermenters.

**M3-35 MALACHITE GREEN; M3-351.**—(1) Prepare:—Glucose 10; 1-1000 malachite green 16 to 25; nutrient agar 1000 of reaction 0·5 per cent acid to phenolphthalein.

**M3-352.**—(1) Prepare:—Peptone 20; lactose 50; glucose 10; nutrose 10; 2 per cent chemically pure malachite green 0·1 N. 1000; hydroxide 15; D. W. 1000.

**M3-353.**—(1) Prepare:—Malachite green 1; lactose 1000.

**Notes.**—<sup>1</sup>Used as a selective medium in differentiation of the *Opportunus* group.

**M3·36 CHINA GREEN: M3·361.**—(1) Prepare :—1·500 China green 15; nutrient agar 1000 of reaction 1·3 per cent acid to phenolphthalein.

**M3·362.**—(1) Prepare :—0·2 per cent china green 15; bouillon 1000.

**M3·37 CAFFEINE: M3·371.**<sup>1</sup>—(1) Prepare : 1 per cent caffeine 1; nutrient medium faintly but permanently alkaline to phenolphthalein 1.

**Notes.**—<sup>1</sup>Used as a selective medium in the differentiation of the typho-coli group before further sowing.

**M3·372.**<sup>1</sup>—(1) Prepare :—Lactose<sup>2</sup> 5; 6 per cent caffeine 110; litmus sol.<sup>2</sup> (S7·783) 60; agar 50; bouillon containing 6 per cent peptone and neutral to litmus, 1000.

**Notes.**—<sup>1</sup>For differentiation of the typho-coli group. <sup>2</sup>Added to the hot sterilized nutrient agar.

**M3·373.** (1) Prepare : 10 per cent caffeine 33; Endo medium (M3·412) 1000.

**M3·38 EOSIN-METHYLENE BLUE: M3·381.**<sup>1</sup>—(1) Prepare and sterilize: Peptone 10; di-pot. phosphate 2; agar 15; D. W. 1000. (2) Add, just before use:—sterile 20 per cent lactose 50; sterile 2 per cent yellow eosin 20; sterile 2 per cent methylene blue 20.

**Notes.**—<sup>1</sup>For cultivation of coli-aerogenes group. *B. aerogenes* is the prevailing coliform organism in soil and on grain. The medium requires no adjustment of reaction.

**M3·39 TELLURIC ACID: M3·391.**<sup>1</sup>—(1) Prepare :—Sterilized sheep serum at 45C., 50; sterile 1 per cent telluric acid at 45C., 9; melted nutrient agar neutral to litmus at 45C., 1000.

**Notes.**—<sup>1</sup>A selective medium for *B. diphtherie*.

**M3·4 INHIBITORY SUBSTANCE: M3·41 SOD. SULPHITE.**

**M 3 411.**—(1) Prepare : Lactose 10; nutrient agar 1000 with reaction 0·5 per cent alkaline to phenolphthalein. (2) Filter. (3) Add alc. fuchsin<sup>1</sup> 5. (4) Mix. (5) Add 10 per cent freshly prepared sod. sulphite 25. (6) Sterilize.

**Notes.**—<sup>1</sup>(i) Prepare :—Basic fuchsin 1; alc., alc. 20. (ii) Allow to stand 24 hr. (iii) Centrifugalize. (iv) Use the S. N. F.

**M3·412.**<sup>1</sup>—(1) Prepare :—10 per cent crystalline sod. bicarbonate 10; nutrient agar neutral to litmus, 1000. (2) Add :—Chemically pure lactose 10; filtered alc. fuchsin,<sup>2</sup> 5. (3) Add 10 per cent freshly prepared

crystalline sod. sulphite 25. (4) Distribute into T. T. or flasks. (5) Sterilize<sup>3</sup> at 115°C. (6) Keep in the dark till use.

**Notes.**—<sup>1</sup>For differentiation of the typho-coli group organisms. <sup>2</sup>Prepare: (i) basic fuchsin 1; abs. alc. 20. (ii) Allow to stand 24 hr. (iii) Centrifugalize. (iv) Use the S.N.F. A filtered sat. alc. fuchsin in the same or half the quantity will serve the purpose. <sup>3</sup>With sterilization the descolourization effected by the sod. sulphite is completed.

**M3\*413.**<sup>1</sup>—(1) Prepare:—10 per cent anhydrous sod. carbonate 3·7; nutrient agar 0·5 per cent acid to phenolphthalein. (2) Prepare No. 1 sol.:—lactose 2 grm.; D. W. 25 c.c. (3) Prepare No. 2 sol. fresh:—anhydrous sod. sulphite 0·5 grm.; sat. alc. basic fuchsin 1 c.c.; D. W. 10 c.c. (4) Add No. 2 sol. to No. 1 sol. and the mixture of the two to 200 c.c. already prepared alkaline nutrient agar. (5) Prepare plates immediately. (6) Dry the surface of the medium for 15 min. in the incubator.

**Notes.**—<sup>1</sup>An Endo medium for cultivation of *B. coli* in the examination of water.

**M3\*414.**<sup>1</sup>—(1) Prepare:—Peptone 10; di-pot. phosphate 3·5; agar 20; D. W. 1000 without any adjustment of reaction or filtration. (2) Add to the melted peptone agar:—20 per cent lactose 50; 10 per cent alc. basic fuchsin 5; freshly prepared 10 per cent sod. sulphite 25. (3) Pour plates. (4) Allow to harden in the incubator with the agar surface downwards. (5) Keep in the dark.

**Notes.**—<sup>1</sup>A rapid method of preparation of Endo medium.

**M3\*415.**<sup>1</sup>—(1) Prepare the solutions:—10 per cent anhydrous sod. carbonate, 20 per cent saccharose, and 20 per cent dextrin. (2) Sterilize these solutions. (3) Prepare the solutions:—Sat. alc. fuchsin and 10 per cent sod. sulphite.<sup>2</sup> (4) Sterilize these solutions once at 100°C. (5) Prepare:—10 per cent anhydrous sod. carbonate at 45°C., 6; melted nutrient agar at 45°C., 100. (6) Steam<sup>3</sup> 15 min. (7) Add to the alkaline agar while hot:—Sterile 20 per cent saccharose 5; sterile 20 per cent dextrin 5; sterile sat. alc. fuchsin 0·25; sterile 10 per cent sod. sulphite 2·5. (8) Place the flasks in a sloping position to allow the precipitate formed to settle. (9) Pour plates carefully, avoiding as far as possible the transference of settled precipitate. (10) Place the plates to dry in the incubator with their agar surface downwards. (11) Preserve in the dark for 3 d. before use.

**Notes.**—<sup>1</sup>For cultivation of *V. cholerae*, by direct plating on test medium. The solution does not keep and must be prepared fresh as required. <sup>2</sup>Prepare:—Sat. alc. fuchsin 1; abs. alc. 20. (iii) Centrifugalize. (iv) Use the S.N.F. A filtered sat. alc. fuchsin in the same or half the quantity will serve the purpose. <sup>3</sup>With sterilization the descolourization effected by the sod. sulphite is completed.

**M3\*42 SOD. SALICYLATE: M3\*421.**<sup>1</sup>—(1) Prepare:—Sod. salicylate 1; nutrient agar 100.

**Notes.**—<sup>1</sup>For cultivation of moulds and bacteria to the exclusion of *B. coli*.

**M3·5 MEAT: M3·51.<sup>1</sup> (1) ALKALINE MEAT** (1) Cut up 500 gm. fat-free ox or horse heart. (2) Add water to just cover the meat. (3) Cook thoroughly and slowly in a closed vessel over a small flame. (4) Pour off the liquid portion into a beaker. (5) Mince the solid residue very finely. (6) Add the minced residue to the liquid in the beaker. (7) Bring up the weight of the contents of the beaker to 1000 gm. (8) Break down any small lumps of meat between the fingers. (9) Make faintly alkaline to litmus. (10) Distribute into T. T. (11) Cover with a layer of paraffin. (12) Sterilize 45 min. at 110 C.

**Notes.**—<sup>1</sup>For cultivation of anaerobes.

**M3·52.**—(1) Make finely minced fat-free beef faintly alkaline to litmus. (2) Sterilize in T. T.

**M3·6 MEAT EXTRACT.<sup>1</sup>**

**Notes.**—<sup>1</sup>The basis used in the preparation of most nutrient media.

**M3·61.**—(1) Proceed as in the preparation of bouillon (**M2·111**) through steps (1) to (7). (2) Add sod. chloride<sup>1</sup> 5 gm. to the filtrate. (3) Steam 45 min. (4) Bring the vol. up to 1000 c.c. by the addition of water. (5) Estimate and adjust the reaction. (6) Steam 30 min. (7) Filter, while hot, through well-wetted, thick, filter paper. (8) Distribute into flasks. (9) Sterilize.

**Notes.**—<sup>1</sup>The addition of sod. chloride is omitted, if it is to be subsequently added in the course of preparation of nutrient medium.

**M3·62.<sup>1</sup>**—(1) Prepare:—Lemco 0·4; sod. chloride 0·4; agar 25; D. W. 1000. (2) Make the reaction 1 per cent alkaline to phenolphthalein.

**Notes.**—<sup>1</sup>For cultivation of protozoa.

**M3·7 MILK, WHEY, CASEIN, AND MILK PRODUCTS:**

**M3·71 MILK: M3·711.**—(1) Steam fresh cow's or goat's milk 60 min. (2) Leave in a cool place or in the ice chest overnight to allow the cream to rise. (3) Skim off the cream which has risen. (4) Transfer the creamed milk in quantities of 10 c.c. to T. T. (5) Sterilize 20 min. at 100C. on each of five successive days. (6) Test sterility by incubation 48 hr.

**M3·72 INDICATOR MILK<sup>1</sup>: M3·721.**—(1). Tinge sterilized creamed milk (**M3·711**) in T. T. with litmus sol. to give a pale blue colour. (2) Sterilize 45 min. 3 d.

**Notes.**—<sup>1</sup>If the purchased milk is acid in reaction, sod. carbonate should be added to neutralize.

**M3·722.**—(1) Prepare :—Creamed milk 1 : water 3. (2) Prepare : China blue-rosolic acid indicator sol.<sup>1</sup> 2·5 : diluted milk 100. (3) Raise to boiling point. (4) Boil 5 min. (5) Adjust the reaction by bringing the colour, with the addition of alkali, to a pale grey. (6) Distribute into T. T. containing gas tubes which should project above the surface. (7) Sterilize in the autoclave.

**Notes.**—<sup>1</sup>Equal parts 1 per cent watery China blue and 0·5 per cent. alc. rosolic acid.

**M3·73 MILK AGAR: M3·731.**—(1) Add 3 c.c. sterilized plain or litmus milk (**M3·721**) to a tube of melted nutrient agar.

**M3·732.**—(1) Prepare :—Sterilized milk at 45°C., 1 : melted nutrient agar at 45°C., 1.

**M3·74 MILK RICE: M3·741.**—(1) Make a paste with :—Rice powder 10 : bouillon 7 : creamed milk 2. (2) Distribute as a layer in T. T. or flasks. (3) Heat over boiling water to solidify the paste. (4) Sterilize 30 min. at 100°C. 3 d.

**M3·742.**—(1) Make a paste with :—Rice powder 2 : bouillon 1 : milk 3. (2) Distribute as a layer in T. T. or flasks. (3) Sterilize 30 min. at 100°C. 3 d.

**M3·743.**—(1) Make a paste with :—Bouillon 1 : powdered rice 2 : milk 3. (2) Distribute as a layer in a Petri dish. (3) Sterilize 20 min. at 115°C.

**M3·75 WHEY: M3·751**—(1) Add rennet to fresh milk. (2) Keep at 60°C. (3) Strain the separated whey through a thick, clean cloth. (4) Make the reaction of the fluid neutral to litmus by means of an organic acid, such as citric acid. (5) Steam 60 min. (6) Filter, while hot, through well-wetted, thick, filter paper. (7) Add litmus sol. to give a deep purple red colour. (8) Distribute into flasks or T. T. (9) Sterilize.

**M3·752.**—(1) Add 1·5 c.c. strong hydrochloric acid or glacial acetic acid to 1000 c.c. fresh, slightly warm milk. (2) Boil. (3) Filter through well-wetted, thick, filter paper. (4) Make the reaction of the filtrate neutral to litmus by the addition of dilute sod. carbonate solution. (7) Filter, while hot, through well-wetted, thick, filter paper. (8) Add litmus sol. to give a deep purple red colour. (9) Distribute into T. T. or flasks. (10) Sterilize.

**M3·753.**<sup>1</sup>—(1) Prepare :—Fresh milk 1000 : 1·4 hydrochloric acid 5. (2) Keep at 37°C. (3) Filter. (4) Make the filtrate neutral to litmus with sod. hydroxide. (5) Steam 2 hr. (6) Estimate acid and adjust the

reaction. (7) Filter. (8) Sterilize at 100°C. (9) Prepare:—Sterilized whey 1; nutrient agar 2.

**Notes.**—<sup>1</sup>For cultivation of gonococcus.

**M3-754.**<sup>1</sup>—(1) Prepare:—25 per cent crystalline calc. chloride<sup>2</sup> 1; fresh milk 100. (2) Heat 5 min. at 115°C. (3) Allow to cool. (4) Strain without shaking or squeezing through a well-wetted, thick cloth. (5) Add litmus sol. to tint the fluid obtained. (6) Bring to the tint desired by the addition of sod. hydroxide sol. (7) Boil 1 min. (8) Allow to cool. (9) Decant the whey. (10) Filter through well-wetted, thick, filter paper. (11) Distribute into T. T. (12) Sterilize at 112°C.

**Notes.**—<sup>1</sup>For differentiation of organisms of the typho-coli group. <sup>2</sup>The use of calc. chloride for the coagulation of the milk obviates the alteration in reaction produced by the addition of an acid.

**M3-76 CASEIN: M3-761.**—(1) Prepare:—Casein 10 gm.; D. W. 100 c.c. (2) Add N-1 sod. hydroxide 7 c.c. (3) Steam until the casein is dissolved. (4) Dissolve 10 gm. agar in 900 c.c. D. W. (5) Mix the agar sol. with the casein sol. (6) Filter through thick, filter paper. (7) Adjust the reaction to 1·5 per cent acid to phenolphthalein. (8) Distribute into T. T. (9) Sterilize in autoclave.

**Notes.**—<sup>1</sup>For cultivation of soil bacteria.

**M3-762.**—(1) Prepare:—Di-pot. phosphate 1; mag. sulphate 0·3 sod. chloride 0·1; calc. chloride 0·1; D. W. 1000. (2) Add, if required, a trace of ferric chloride and also, if required, 1 per cent peptone. (3) Make faintly alkaline to litmus. (4) Add casein 0·5 per cent. (5) Heat in a water bath with shaking to distribute the casein. (6) Sterilize 15 min. at 115°C. (7) Filter. (8) Add 1 per cent sugar. (9) Sterilize at 100°C. (10) Distribute into T. T.

**M3-763<sup>1</sup> ALKALINE CASEIN.**—(1) Prepare:—Casein<sup>2</sup> 20; N-1 sod. hydroxide 10; water 1,000.

**Notes.**—<sup>1</sup>For cultivation of anaerobic organisms. <sup>2</sup>Or nutrose or other casein preparation.

### **M3-77 DRIED MILK PRODUCTS.<sup>1</sup>**

**Notes.**—<sup>1</sup>There are a great variety of these which are usable in the preparation of media. Some of them are simply dried milk and may be used as milk or casein is used. Casein combined with ammonia is sold as "encasein" and casein with potassium as "plasmon." "Sanose" is said to contain 80 per cent of casein and 20 per cent of egg albumin. "Sanatogen" consists of casein and 5 per cent glycono-phosphate. "Nutrose" contains casein, sod. carbonate, flour and vegetable protein derived from the pea-nut.

**M3-771.**<sup>1</sup>—(1) Prepare :—Nutrose<sup>2</sup> 15 ; agar 15 ; water 1000.

**Notes.**—<sup>1</sup>For cultivation of amœbæ. <sup>2</sup>Or somatose.

**M3-772.**—(1) Prepare No. 1 sol. :—Nutrose 1 ; sol. chloride 0.5 ; water 75. (2) Prepare No. 2 sol. :—Sugar 1 ; water 25 ; litmus sol. to give an amethyst tint. (3) Mix No. 1 and No. 2 sol. after cooling. (4) Distribute in T. T. (5) Test sterility by incubation 48 hr.

**M3-8 ORGANS, TISSUES : M3-81 ORGAN OR ORGAN FRAGMENT : M3-811.**<sup>1</sup>—(1) Kill a rabbit. (2) Fasten it on a P. M. board on its back with limbs fully extended and pinned to the board. (3) Wet the hair over thorax and abdomen with 2 per cent lysol or other antiseptic. (4) Cut the hair as short as possible and again wet the body surface with antiseptic sol. (5) Make a mesial incision with sterile instruments through the skin, to extend over thorax and abdomen. (6) Dissect off the skin in flaps to expose the anterior surface of thorax and abdomen completely. (7) Use a fresh set of sterile instruments and open the abdomen. (8) Remove with sterile precautions the organs,<sup>2</sup> liver, kidneys, spleen and testicles. (9) Place in sterile Petri dishes. (10) Open the thorax<sup>3</sup> with a fresh set of sterile instruments. (11) Remove the lungs and place in sterile Petri dishes. (12) Cut up the organs obtained with sterile precautions.

**Notes.**—<sup>1</sup>Used largely in the way of addition to media for the cultivation of spirochetes and anaerobic organisms. <sup>2</sup>Organs and tissues obtained at ordinary post-mortem examination may also be used, after sterilization. <sup>3</sup>If the thorax as well as the abdomen is to be opened, the former should be opened first.

**M3-812.**—(1) Remove with sterile precautions the organs lungs, liver, kidneys, spleen, and testicle from a rabbit specially killed for the purpose (M3-811). (2) Scar the surface of the organ. (3) Cut out suitably sized portions with sterile forceps and scissors. (4) Place in sterile T. T. or Petri dishes.

**M3-813.**<sup>1</sup>—(1) Distribute sterile peptone water 2 per cent. acid to phenolphthalein in quantities of 10 c.c. in T. T. (2) Add about 1 gm. fresh rabbit, ox, or human liver. (3) Sterilize 15 min. at 115C. (4) Test sterility by incubation 46 hr.

**Notes.**—<sup>1</sup>For cultivation of streptococci, and anaerobic organisms.

**M3-814.**<sup>1</sup>—(1) Use sterile T. T. 20 × 2 cm. (2) Transfer small portions<sup>2</sup> of organs sterily removed from a rabbit specially killed for the purpose (M3-811), to each T. T. (3) Prepare :—(16) sterile conditions :—Melted nutrient agar 1 per cent acid to phenolphthalein at 45C., 1 ; sterile ascitic fluid at 45C., 1. (4) Fill with sterile peptone water.

about 20 c.c. of ascitic fluid agar into each T. T. (5) Cover the medium to a depth of 3 cm. with sterile liquid paraffin. (6) Test sterility by incubation 48 hr.

**Notes.** —<sup>1</sup>For cultivation of anaerobic spirochetes. <sup>2</sup>About 4 mm. cubes.

**M3·815.**<sup>1</sup>—(1) Treat cut up portions of organs sterily removed from a rabbit specially killed for the purpose (**M3·811**) with 6 per cent glycerinated 0·85 S. S. S. for 1 hr. (2) Prepare sterile T. T. containing a 2 cm. length of glass tubing as support for the tissue. (3) Add 6 per cent glycerinated 0·85 S. S. S. to the T. T. to the top of the supporting glass rod. (4) Transfer a suitably sized portion of glycerinated organ tissue to each T. T. to rest on the glass tubing support. (5) Sterilize 30 min. at 120°C.

**Notes.** —<sup>1</sup>For cultivation of *B. tuberculosis*.

**M3·82 ORGAN EXTRACT: M3·821.**—(1) Mince finely lungs, liver, spleen, kidney, testicles, thymus, placenta, or other organ. (2) Proceed as for the preparation of bouillon.

**M3·84 BRAIN: M3·841.**<sup>1</sup>—(1) Make a pulp of fresh brain. (2) Add 500 grm. pulp to 500 c.c. D. W. (3) Raise the temperature slowly to boiling point with constant stirring. (4) Boil 15 min. (5) Strain through cloth to give a pulpy mass. (6) Steam 2 hr. (7) Prepare:—Brain pulp mass 1; serum 1. (8) Add 3 per cent glycerin. (9) Distribute into T. T. (10) Coagulate (**M6·1**).

**Notes.** —<sup>1</sup>For cultivation of *B. tuberculosis*.

**M3·85 TESTICLE: M3·851.**<sup>1</sup>—(1) Remove the tunica vaginalis of bulls' testicles. (2) Mince finely. (3) Allow to macerate in water overnight. (4) Heat in a water bath<sup>2</sup> with constant stirring until the proteins have been coagulated. (5) Filter through coarse cloth. (6) Make up to vol. 750 c.c. with hot D. W. (7) Add to the fluid, while hot, 20 grm. peptone and 3 grm. di-hydrogen sod. phosphate. (8) Allow the temperature to fall to 40C. (9) Estimate and adjust the reaction to pH 7·6. (10) Add 30 grm. agar previously dissolved in 250 c.c. D W. and finally 5 grm. glucose. (11) Mix. (12) Distribute in T. T. (13) Sterilize at 120C. (14) Rotate the tubes to mix before allowing to solidify.

**Notes.** —<sup>1</sup>For cultivation of gonococci. <sup>2</sup>Not over a free flame.

**M3·9 PEPSIN DIGEST: M3·91 SOLUTION: M3·911.**—(1) Clean and wash a number of pigs' stomachs. (2) Mince finely.

(3) Prepare.—Minced stomach 200; strong hydrochloric acid 10; water at 50C. 1000. (4) Keep the mixture at 50C. 20 hr. (5) Raise the temperature to boiling point. (6) Pour the mixture on to a thick, clean, cloth. (7) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth and its contents. (8) Heat the fluid collected to 80C. (9) Make the reaction faintly alkaline to litmus at 80C. (10) Filter, while hot, through well-wetted, thick, filter paper. (11) Steam 30 min. (12) Filter again through well-wetted, thick, filter paper. (13) Distribute the filtrate into flasks or T. T. (14) Sterilize.

**M3-92 BOUILLON: M3-921.**<sup>1</sup>—(1) Mince finely fat-free veal. (2) Add 500 grm. to 1000 c.c. water. (3) Place 18 hr. at 37C. (4) Pour the mixture on to a thick, clean cloth. (5) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (6) Mix the fluid collected with an equal vol. of pepsin digest sol. (**M3-911**). (7) Heat to 70C. (8) Make the reaction neutral to litmus. (9) Add 7 c.c. N-1 sod. hydroxide per litre. (10) Filter through well-wetted, thick, filter paper. (11) Steam 30 min. (12) Filter, while hot, through well-wetted, thick, filter paper. (13) Distribute the filtrate into flasks or T. T. (14) Sterilize.

**Notes.**—<sup>1</sup>Pepsin digest bouillon. Solidify with agar to make pepsin digest agar.

**M3-922.**<sup>1</sup>—(1) Prepare:—Minced pigs' stomach 10; minced ox or pig liver 10; strong hydrochloric acid 1; water 100. (2) Digest 24 hr. at 50C. (3) Boil. (4) Decant after 24 hr. the S. N. F. (5) Make faintly alkaline to litmus. (6) Distribute into T. T. or flasks.

**Notes.**—<sup>1</sup>For blood culture. Sow 5 c.c. blood into 50 c.c. bouillon.

**M3-923.**—(1) Mince finely pig or beef liver, fat-free beef, placenta, blood clot. (2) Wash clean and mince finely a number of large pigs' stomachs. (3) Prepare:—Minced liver, beef, placenta or blood clot 100; minced stomachs 100; strong hydrochloric acid 10; T. W. at 50C. 1000. (4) Keep the mixture 20 hr. at 50C. (5) Make a biuret<sup>1</sup> and a tryptophane test.<sup>2</sup> (6) Boil 10 min. to stop the digestion. (7) Pour the mixture on to a wet, thick, clean cloth. (8) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (9) Filter the fluid collected through well-wetted, thick, filter paper. (10) Bring the vol. up to 1000. (11) Filter through well-wetted, thick, filter paper. (12) Add 0.2 per cent di-pot. phosphate. (13) Estimate and adjust the reaction. (14) Steam 30 min. (15) Filter

while hot, through well-wetted, thick, filter paper. (16) Distribute into flasks or T. T. (17) Sterilize.

**Notes.**—<sup>1</sup>Add to 5 c.c. filtered digest 0.1 c.c. 5 per cent copper sulphate and follow with 5 c.c. N-1 sod. hydroxide. A pink colour indicates complete peptonization. <sup>2</sup>Add slowly bromine water to a filtered sample of the digest rendered acid with acetic acid when, if tryptophane is present, a rose red colour will be produced which may be extracted with amyl. alc. The colouring matter turns to yellow on adding an excess of bromine. The presence of but a slight positive reaction indicates that the digestive process has been carried far enough.

**M3-924.**<sup>1</sup>—(1) Prepare: Glucose 15; pot. nitrate 2; agar 3; meat extract (M3-61) prepared from veal 500; pepsin digest bouillon (M3-921) 500.

**Notes.**—<sup>1</sup>For cultivation of *B. diphtheria*.

**M3-93 BLOOD: M3-931.**—(1) Procure ox or sheep blood from the slaughterhouse. (2) Allow to clot. (3) Decant and store the serum in an ice chest. (4) Mince the clot. (5) Add 100 gm. clot to 1000 c.c. T. W. (6) Raise slowly to boiling temperature. (7) Boil 10 min. (8) Cool to 50 C. (9) Add 100 gm. minced pigs' stomach and 10 c.c. strong hydrochloric acid. (10) Digest 20 hr. (11) Proceed further as in steps (5) to (12) in M3-923. (12) Make the reaction neutral to litmus. (13) Add 75 c.c. of the decanted serum. (14) Steam 60 min. (15) Allow the clot to form a compact mass. (16) Decant the clear fluid. (17) Sterilize at 100C.

**M3-932.**<sup>1</sup>—(1) Prepare by addition in the order given: 0.85 S. S. S. 150 c.c.; hydrochloric acid 6 c.c.; defibrinated sheep's blood 50 c.c.; granulated pepsin B. P. 1 gm. (2) Shake to dissolve. (3) Heat in the water bath at 55C. for 2 to 24 hr.<sup>2</sup> with occasional shaking. (4) Add 12 c.c. 20 per cent sod. hydroxide. (5) Adjust the reaction by addition of 20 per cent sod. hydroxide until a sample gives with cresol red indicator sol.<sup>3</sup> the colour of permanganate.<sup>4</sup> (6) Add pure hydrochloric acid drop by drop until cresol red indicator sol. gives practically no change of colour but phenol red gives red.<sup>5</sup> (7) Add chloroform to 0.25 per cent. (8) Shake to mix. (9) Keep in a tightly stoppered bottle till required for use. (10) Prepare for use:—Peptic digest blood at 45C., 3.5; melted nutrient agar at 45C., 100.

**Notes.**—<sup>1</sup>For the cultivation of *B. influenza*. <sup>2</sup>The exact time is immaterial. <sup>3</sup>0.02 per cent. <sup>4</sup>Corresponding to pH 7.6. <sup>5</sup>Corresponding to pH 7.0 to 7.2.

**M3-94 PEPSIN TRYPSIN DIGEST: M3-941.**—(1) Prepare:—Finely minced horse heart 150 gm.; pepsin 5 gm.; 50 per cent hydrochloric acid 2 c.c. D. W. 400 c.c. (2) Leave to digest 2 d. at 37C.

(3) Filter. (4) Add anhydrous sod. carbonate 3.9 gm. and 15 c.c. fresh glycerin pancreatic extract (**T7.4**). (5) Leave 6 hr. at 37°C. (6) Sterilize at 100°C. (7) Neutralize with hydrochloric acid. (8) Add water 1950 c.c.; sod. chloride 6 gm.; agar 39 gm. (9) Steam 3 hr. (10) Filter. (11) Distribute into T. T. and flasks. (12) Sterilize.

**M3-942.** (1) Proceed as in steps (1) to (10) in **M3-923**. (2) Make faintly alkaline to litmus with sod. carbonate sol. (3) Cool to 37°C. (4) Add pancreatic extract to 1 per cent. (5) Keep at 37°C. 3 hr. or longer. (6) Make tests for tryptophane.<sup>1</sup> (7) Make slightly acid to litmus with glacial acetic acid. (8) Raise slowly to boiling point. (9) Boil 10 min. (10) Filter, while hot, through well-wetted, thick, filter paper. (11) Add 0.2 per cent di pot. phosphate. (12) Estimate and adjust the reaction. (13) Steam 30 min. (14) Filter, while hot, through well-wetted, thick, filter paper. (15) Distribute into flasks or T. T. (16) Sterilize.

**Notes.**—<sup>1</sup>**M3-9232.**

#### **M4 MEDIA PREPARATION AND COMPOSITION: M4-1 PEPTONE AND PEPTONE DERIVATIVES.<sup>1</sup>**

**Notes.**—<sup>1</sup>Tryptophane, polypeptids amino-acids, etc. The peptone media here dealt with are those without the addition of meat extract.

**M4-11.<sup>1</sup>**—(1) Prepare:—Sod. chloride 5; peptone 10; D. W. 100. (2) Boil to dissolve the peptone. (3) Add:—lactose 10; sod. neutral red 10. (4) Heat to dissolve the sugar. (5) Filter. (6) Distribute in T. T. containing fermentation tubes. (7) Sterilize 3 d. at 100°C.

**Notes.**—<sup>2</sup>Solidify with agar if required.

**M4-12.<sup>1</sup>**—(1) Prepare:—Sod. chloride 5; peptone 10 to 20; D. W. 1000.

**Notes.**—<sup>1</sup>Peptone water. Solidify with agar if desired. With reaction pH 8 to pH 9 if used for isolation of *V. cholerae*.

**M4-13.**—(1) Prepare:—Sugar 40; peptone 10 to 20; D. W. 1000. (**S7-78**) 50; D. W. 1000.

**M4-14.**—(1) Prepare:—Granulated peptone of Chassaing 2; pure anhydrous glycerin 1; agar 2; D. W. 100.

**M4-15.**—(1) Prepare:—Peptone 10; glucose 40; agar 20; water 1000, 2 per cent acid to phenolphthalein.

**Notes.**—<sup>2</sup>For cultivation of yeast.

**M4-16.<sup>1</sup>**—(1) Prepare:—peptone 15; glucose 1; yeast 0.5; sod. chloride 0.5; agar 2; water 10 without any adjustment of reaction.

**Notes.**—<sup>1</sup>For cultivation of hyphomycetes.

**M4·17.**<sup>1</sup>—(1) Prepare :—Peptone 3 ; agar 1·8 ; water 100.

**Notes.**—<sup>1</sup>For cultivation of hyphomycetes.

**M4·18.**<sup>1</sup>—(1) Prepare : di-pot. phosphate 5 ; mag. sulphate 2·5 ; sod. chloride 2·5 ; peptone<sup>2</sup> 1 ; D. W. 1000.

**Notes.**—<sup>1</sup>For demonstration of ammonia production from protein. <sup>2</sup>Or fibrin or gelatin.

#### **M4·2 PROTEIN FREE.**<sup>1</sup>

**Notes.**—<sup>1</sup>Often called "synthetic" media. The idea conveyed by the term synthetic is that the media are composed of substances of known chemical composition. See also media containing salts of mineral and organic acids. Ammonium salts can afford the nitrogen, and "sugars" the carbon required for the metabolism of bacteria.

**M4·21 : M4·211.**—(1) Prepare : Asparagin 3·4 ; ammon. lactate 10 ; sod. chloride 5 ; mag. sulphate 0·2 ; calc. chloride 0·1 ; di-pot. phosphate 1 ; glycerin 10 ; D. W. 1000.

**M4·212.**—(1) Prepare : Sod. chloride 5 ; calc. chloride 0·1 ; mag. sulphate 0·2 ; di-hydrogen pot. phosphate 2 ; ammon. lactate 6 ; pot. aspartate<sup>1</sup> 3 ; glycerin 30 ; D. W. 1000.

**Notes.**—<sup>1</sup>Or sod. aspartate.

**M4·213.** (1) Prepare : Asparagin 1 ; di-sod. phosphate 2 ; ammon. lactate 6 ; sod. chloride 5 ; D. W. 1000.

**M4·214.**—(1) Prepare :—Sod. chloride 2 ; mag. sulphate 0·1 ; calc. chloride 0·2 ; di-hydrogen pot. phosphate 2 ; asparagin 2 ; mannite 2 ; water 1000. (2) Make reaction neutral to litmus. (3) Filter. (4) Add litmus sol. 17·5. (5) Distribute in T. T. (6) Sterilize.

**M4·215.**—(1) Prepare No. 1 sol. :—Pot. nitrate 2 gm. ; asparagin 0·1 gm. ; D. W. 25 c.c. (2) Prepare No. 2 sol. :—Citric acid 0·5 gm. ; di-hydrogen pot. phosphate 0·2 gm. ; mag. sulphate 0·2 gm. ; calc. chloride 0·02 gm. ; ferric chloride a trace ; D. W. 50 c.c., and make neutral to litmus with pot. hydroxide sol. (3) Mix Nos. 1 and 2 solutions and make up to 100 c.c. (4) Distribute into T. T. (5) Sterilize.

**M4·216.**<sup>1</sup>—(1) Prepare :—Asparagin 5 ; ammon. lactate 5 ; sod. sulphate 2 ; mag. sulphate 0·2 ; D. W. 1000.

**Notes.**—<sup>1</sup>Use as such or for indol test with the addition of either peptone 30, or 1-1000 tryptophane 1, 3, or 5.

**M4·217.**<sup>1</sup>—(1) Prepare :—Sod. asparaginate 1 ; glucose 1 ; glycerin 10 ; di-hydrogen ammon. phosphate 1·5 ; calc. chloride 0·1 ; mag. sulphate 0·2 ; pot. chloride 0·1 ; ferric chloride a trace ; agar 12 ; D. W. 1000 with reaction 1 per cent acid to phenolphthalein.

**Notes.**—<sup>1</sup>For cultivation of soil bacteria.

**M4·218.**—(1) Prepare :—Sod. chloride 5 ; calc. chloride 0·1 ; mag. sulphate 0·3 ; di-pot. phosphate 2·5 ; ammon. lactate 8 ; sod. asparaginate 3·5 ; glycerin 35 ; water 1000.

**M4·219.**—(1) Prepare :—Di-hydrogen pot. phosphate 0·5 ; mag. sulphate 0·06 ; mag. citrate 0·25 ; asparagin 0·5 ; glycerin 2 ; sod. hydroxide 0·25 ; water 100.

**M4·22 : M4·221.**—(1) Prepare :—Di-pot. phosphate 1 ; mag. sulphate 0·5 ; ammon. sulphate 1 ; sod. chloride 0·06 ; precipitated chalk 20 ; D. W. 1000.

**M4·222.**—(1) Prepare :—Ammon. tartrate 10 ; di-hydrogen pot. phosphate 5 ; calc. phosphate 0·5 ; mag. sulphate 5 ; D. W. 1000.

**M4·223.**—(1) Prepare :—Ammon. tartrate 10 ; di-pot. phosphate 1 ; mag. sulphate 0·2 ; calc. chloride 0·1 ; D. W. 1000.

**M4·224.**—(1) Prepare :—Ammon. nitrate 1 ; ammon. phosphate 0·6 ; ammon. sulphate 0·25 ; tartaric acid 1 ; pot. carbonate 0·6 ; zinc sulphate 0·07 ; ferrous sulphate 0·07 ; pot silicate 0·07 ; saccharose 70 ; water 1500.

**M4·225.**—(1) Prepare :—Ammon. tartrate 10 ; di-pot. phosphate 10 ; mag. sulphate 10 ; tri-calc. phosphate 0·5 ; D. W. 1000.

**M4·226.**—(1) Prepare :—Ammon. tartrate 10 ; di-pot. phosphate 1 ; mag. sulphate 0·2 ; calc. chloride 0·12 ; water 1000.

**M4·227.**—(1) Prepare :—Ammon. acetate 10 ; di-pot. phosphate 5 ; mag. sulphate 5 ; tri-basic calc. phosphate 0·5 ; D. W. 1000.

**M4·228.**<sup>1</sup>—(1) Prepare :—Ammon. carbonate 3·5 ; di-hydrogen pot. phosphate 1·3 ; mag. sulphate 2·5 ; glycerin 15 ; water 1000.

**Notes.**—<sup>1</sup>For cultivation of *B. tuberculosis*.

**M4·23 : M4·231.**—(1) Prepare :—Ammon. sulphate 1 ; sod. chloride 2 ; ferrous sulphate 0·4 ; mag. sulphate 0·5 ; di-pot. phosphate 1. (2) Sterilize. (3) Add mag. carbonate in excess.

**M4·232.**<sup>1</sup>—(1) Prepare :—Ammon. nitrate 4·5 ; saccharose 70 ; neutral pot. tartrate 6·5 ; di-pot. phosphate 0·6 ; mag. carbonate 0·4 ; pot. sulphate 0·25 ; ferrous sulphate 0·07 ; zinc sulphate 0·07 ; pot. silicate 0·07 ; D. W. 1500.

**Notes.**—<sup>1</sup>For cultivation of *B. anthracis*.

**M4·233.**<sup>1</sup>—(1) Prepare :—Sod. monophosphate 0·1 ; di-pot. phosphate 0·1 ; lactose 0·5 ; agar 1 ; D. W. 1000. **M4·234.**—(1) Prepare :—Sod. monophosphate 0·1 ; di-pot. phosphate 0·1 ; lactose 0·5 ; agar 1 ; D. W. 1000.

mixture at 90°: 1 per cent. alc. basic fuchsin 0.5; freshly prepared 5 per cent. sod. sulphite 0.5.

**Notes.**—<sup>1</sup>For cultivation of *coli-aerogenes* group: allowing only the development of organisms capable of taking their nitrogen from a very simple source and of utilizing lactose as their only source of carbon.

**M4.234.**—(1) Prepare:—Sod. chloride 0.002; mag. sulphate 0.002; di-pot. phosphate 0.05; ferric chloride a trace; glycerin<sup>1</sup> 1; urea<sup>2</sup> 0.1; D. W. 100.

**Notes.**—<sup>1</sup>As other sources of carbon, saccharose, glucose, mannite, calc. lactate, etc., are used. <sup>2</sup>As other sources of nitrogen, sod. hippurate, uric acid, asparagin, ammon. sulphate, ammon. acetate, ammon. butyrate, ammon. lactate, are used.

**M4.235.**—(1) Prepare:—Sod. nitrate 0.1; sod. chloride 0.002; mag. sulphate 0.002; di-pot. phosphate 0.05; ferric chloride a trace; glycerin or glucose 1; D. W. 100.

**M4.236.**—(1) Prepare:—Glycoroll 1; arginine 0.5; sod. chloride 1.25; di-hydrogen pot. phosphate 1.25; mag. citrate 0.6; glucose 1; glycerin 10; water 250. (2) Make neutral to litmus. (3) Add N-100 sod. hydroxide.

**Notes.**—<sup>1</sup>For cultivation of *B. tuberculosis*.

**M4.237.**—(1) Prepare:—Uric acid 0.5; sod. chloride 5; mag. sulphate 0.2; calc. chloride 0.1; di-pot. phosphate 1; glycerin 30; ammonia-free D. W. 1000.

**Notes.**—<sup>1</sup>For cultivation *B. aerogenes* to the exclusion of *B. coli*.

**M4.238.**—(1) Prepare:—Ammonia 0.62; pot. hydroxide 5.6; sod. hydroxide 4; phosphoric acid 12.79; acetic acid 1.13; ferric chloride 0.001; mag. sulphate 0.001; calc. chloride 0.001; water 1000 of pH 7.

**M4.239.**—(1) Prepare:—Pot. nitrate 7; di-pot. phosphate 1.5; mag. sulphate 1.5; sod. chloride 1.5; calc. sulphate 5; ferric chloride sol. a few drops; D. W. 3000 c.c.

**M4.24 : M4.241.**<sup>1</sup>—(1) Prepare:—Glucose 2; citric acid 5; mag. sulphate 2; di-pot. phosphate 2; calc. chloride 0.2; ferric chloride, a trace; D. W. 1000 with strictly neutral reaction.

**Notes.**—<sup>1</sup>For cultivation of aerobic nitrogen-fixing bacteria. Nitrogen-free medium.

**M4.242.**<sup>1</sup>—(1) Prepare:—Mannite 15; mag. sulphate 0.2; di-hydrogen pot. phosphate 0.2; sod. chloride 0.2; calc. sulphate 0.1; calc. carbonate 5; D. W. 1000. (2) Dissolve the phosphate separately in a little water and make neutral to phenolphthalein. (3) Add the other ingredients.

**Notes.**—<sup>1</sup>A nitrogen-free medium. For cultivation of aerobic nitrogen-fixing bacteria and protozoa.

**M4-243.**<sup>1</sup>—(1) Boil 2 hr.:—Rich garden soil 1; water 2. (2) Pour off the turbid fluid. (3) Mix talc<sup>2</sup> with the turbid fluid. (4) Filter and refilter till clear, through thick, filter paper.

**Notes.**—<sup>1</sup>"Soil extract." <sup>2</sup>Or calc. carbonate.

**M4-244.**—(1) Dissolve agar 15 grm. in 900 c.c. water. (2) Add soil extract (**M4-243**) 100 c.c.<sup>1</sup> (3) Add glucose 1 grm. just before distribution into T. T.

**Notes.**—<sup>1</sup>The reaction should be 0.5 per cent. acid to phenolphthalein or nearly neutral.

**M4-245.**<sup>1</sup>—(1) Prepare:—Di-pot. phosphate 0.5; soil extract (**M4-243**) 100; T. W. 900.

**Notes.**—<sup>1</sup>For cultivation of flagellates and ciliates in soil.

**M4-246.**<sup>1</sup>—(1) Prepare:—Ammon. sulphate 2; di-pot. phosphate 1; sod. chloride 2; mag. sulphate 0.5; ferrous sulphate 0.4; D. W. 1000. (2) Distribute in flasks in quantities of 50 c.c. (3) Add to each flask 0.5 grm. mag. carbonate. (4) Sow. (5) Incubate at 30°C.

**Notes.**—<sup>1</sup>For test soil bacteria for ammonia, nitrite, and nitrate production.

**M4-3 PROTEIN POOR: M4-31.**<sup>1</sup>—(1) Prepare: Gelatin 1; T. W. 5 with reaction 0.5 per cent. acid to phenolphthalein.

**Notes.**—<sup>1</sup>Used in soil bacteriology.

#### **M4-4<sup>1</sup> SALTS OF ORGANIC AND INORGANIC ACIDS.**

**Notes.**—<sup>1</sup>See also protein-free media.

**M4-41 INORGANIC SALTS: M4-411.**<sup>1</sup>—(1) Prepare: Di-pot. phosphate 0.5; mag. sulphate 0.2; pot. nitrate 0.06; glucose 10; peptone 0.5; agar 20; D. W. 1000 with reaction 0.5 per cent. acid to phenolphthalein.

**Notes.**—<sup>1</sup>For cultivation of soil organisms.

**M4-12.**<sup>1</sup>—(1) Prepare:—Di-pot. phosphate 1; mag. sulphate 0.2; sod. chloride 0.02; 1:1000 manganese sulphate 1; 1:1000 ferrous sulphate 1; glucose 20; D. W. 1000.

**Notes.**—<sup>1</sup>For cultivation of nitrogen-fixing organisms.

**M4-413.**—(1) Substitute mannite for glucose in **M4-412**.

**M4-414.**<sup>1</sup>—(1) Prepare:—Di-pot. phosphate 1; mag. sulphate 0.5; calc. chloride 0.01; sod. chloride 2; D. W. 1000. (2) Distribute into flasks in quantities of 20 c.c. (3) Add a small quantity of freshly washed mag. carbonate. (4) Sterilize 20 min. at 100°C. 3 d. (5) Add with sterile precautions, to each flask 2 c.c. 2 per cent. sodium sulphate. (6) Test sterility before use by incubation 48 hr.

**Notes.**—<sup>1</sup>For demonstration of nitrate and nitrite formation.

**M4415.**<sup>1</sup>—(1) Prepare: Ammon. sulphate 1; pot. sulphate 1; mag. carbonate 7.5; D. W. 1000.

**Notes.**—<sup>1</sup>For demonstration of nitrite formation by nitrifying organisms.

**M4416.**<sup>1</sup>—(1) Prepare:—Sod. nitrite 1; di-pot. phosphate 0.5; mag. sulphate 0.3; sod. chloride 0.5; ferrous sulphate 0.4; anhydrous sod. carbonate 0.3; D. W. 1000.

**Notes.**—<sup>1</sup>For demonstration of nitrate formation by nitrifying organisms.

**M4417.**<sup>1</sup>—(1) Prepare No. 1 sol.:—Ammon. sulphate 0.4; mag. sulphate 0.05; calc. chloride 0.01; D. W. 50. (2) Prepare No. 2 sol.:—Pot. phosphate 0.1; sod. carbonate 0.6; D. W. 50. (3) Prepare No. 3 sol.:—Silicic acid 3.4; D. W. 100. (4) Mix—No. 1 and No. 2 sol.<sup>2</sup> in equal quantities. (5) Add by degrees this mixture to No. 3 sol. with constant stirring until solidification occurs. (6) Distribute the solidified medium in plates. (7) Sterilize 30 min. at 100C. 3 d.

**Notes.**—<sup>1</sup>Silicate jelly. <sup>2</sup>The sterile nutrient mixture of Nos. 1 and 2 sol. may first be inoculated, and then added with sterile precautions to sterilized silicic acid (No. 3 sol.) in a Petri dish. Rotate to mix and allow to solidify.

**M4418.**<sup>1</sup>—(1) Place 4 c.c. sod. silicate<sup>2</sup> sol. in a porcelain dish. (2) Add 10 c.c. concentrated hydrochloric acid to precipitate the silicic acid. (3) Evaporate to dryness. (4) Moisten the silicic acid again with hydrochloric acid. (5) Evaporate to dryness a second time. (6) Wash and transfer to Swedish filter paper the weight of whose ash is known. (7) Wash the precipitate until the water gives no cloudiness with silver nitrate sol. (8) Burn the filter with the precipitate upon it in a weighed platinum or porcelain crucible and heat to redness. (9) Allow to cool and weigh the silicic anhydride residue. (10) Calculate the amount of silicic anhydride in 1 c.c. of the original silicate sol. (11) Dilute the silicate sol. to contain 5 per cent silicic anhydride. (12) Prepare hydrochloric acid of such strength that 1 c.c. just neutralizes 1 c.c. of the sod. silicate sol. using methyl orange as an indicator. (13) Add 104 c.c. of the hydrochloric acid<sup>3</sup> to 100 c.c. of the sod. silicate sol. (14) Distribute into T. T. in amount of 15 c.c. (15) Sterilize 15 min. at 120C. (16) Sow. (17) Add 0.05 c.c. ammon. sulphate sol.<sup>4</sup> per c.c. and a drop or two of sod. carbonate sol. to make the whole alkaline. (18) Pour plates<sup>5</sup> immediately and allow to solidify.

**Notes.** <sup>1</sup>For isolation in pure state of nitrifying organisms. <sup>2</sup>Water glass. <sup>3</sup>The excess of acid prevents coagulation during the subsequent sterilization. <sup>4</sup>Ammon. sulphate 2; di-pot. phosphate 1; sod. chloride 2; mag. sulphate 0.3; ferrous sulphate 0.4; D. W. 1000. The silicic acid sol. gelatinizes rapidly on admixture with the ammon.

sulphat sod. growing may also be performed on the surface of the medium after setting.

**M4·419.**<sup>1</sup>—(1) Prepare: Pure sod. nitrate 2; anhydrous sod. carbonate 1; di pot. phosphate 0·2; agar 15; T. W. 1000.

**Notes.**—<sup>1</sup>For growing nitrate producing organisms.

**M4·42 CALCIUM SALTS: M4·421.**—(1) Prepare: Calc. carbonate<sup>1</sup> 5 to 20; "sugar" 10; bouillon 1000.

**Notes.**—<sup>1</sup>The acid formed by decomposition of the sugar acts on the carbonate with evolution of carbonic acid gas and neutralization of the acid.

**M4·43 IRON SALTS: M4·431.**—(1) Prepare: Ferric tartrate 1; bouillon 1000.

**M4·432.**—(1) Prepare: Sod. chloride 5; di-pot. phosphate 3; peptone 30; water 1000. (2) Distribute in quantities of 10 c.c. into T. T. (3) Add<sup>1</sup> 0·1 c.c. 2 per cent neutral ferric tartrate to each T. T. (4) Sterilize.

**Notes.**—<sup>1</sup>A yellowish precipitate forms.

**M4·44 LEAD SALTS: M4·441.**—(1) Prepare: Lead acetate 1; bouillon 1000.

**M4·442.**—(1) Prepare:—Sod. chloride 5; di-sod. phosphate 3; peptone 30; water 1000. (2) Distribute in quantities of 10 c.c. into T. T. (3) Add 0·1 c.c. 1 per cent neutral lead acetate to each T. T.

**M4·443.**<sup>1</sup>—(1) Add<sup>1</sup> 1 drop (0·05 c.c.) freshly prepared 1-10 lead acetate at 45C. to 4 c.c. melted nutrient agar at 45C. in T. T. (2) Mix. (3) Sow by passing the platinum loop between the glass and the agar.

**Notes.**—<sup>1</sup>For differentiation of organisms of the typhoid group. B. typhosa and B. paratyphosa, B. cause blackening, B. paratyphosa A. and B. (a) cause no blackening.

**M4·444.**—(1) Prepare: Sterile 30 per cent glucose at 45C. 4 drops; sterile 5 per cent lead subacetate at 45C. 2 drops; melted nutrient agar at 45C. 10 c.c.

**M4·45 NITRATES: M4·451.**—(1) Prepare: Pot. nitrate 5; bouillon 1000.

**Notes.**—<sup>1</sup>For test of nitrate reduction by denitrifying organisms.

**M4·452.**—(1) Prepare: Sod. nitrate 1; peptone 10; ammonium chloride 1; W. 1000.

**M4·453.**<sup>1</sup>—(1) Prepare and distribute same as M4·452. (2) For fermentation tubes: Pot. nitrate 2; peptone 1; water 1000.

**Notes.**—<sup>1</sup>For demonstration of denitrifying organisms.

**M4:454.**—(1) Prepare :—Nitrite-free pot. nitrate 2; peptone 1; ammonia free D. W. 1000.

**M4:455.**<sup>1</sup>—(1) Prepare :—Sod. nitrate 2; citric acid 5; mag. sulphate 2; di-pot. phosphate 2; calc. chloride 0·2; water 1000. (2) Neutralize with sod. carbonate sol. (3) Add a trace of ferric chloride.

**Notes.**—<sup>1</sup>For cultivation of denitrifying species.

**M4:456.**<sup>1</sup>—(1) Prepare :—Glucose 1·5; sod. nitrate 0·3; di-pot. phosphate 0·1; T. W. 100.

**Notes.**—<sup>1</sup>For determination of nitrifying power of soil.

### **M4:5 SUGAR.**<sup>1</sup>

**Notes.**—<sup>1</sup>The term is used to include carbohydrates generally, alcohols, glucosides, etc.

**M4:51 SUGAR: M4:511.**<sup>1</sup>—(1) Prepare :—Sod. chloride 0·5; peptone 1; water 100. (2) Steam to dissolve. (3) Make faintly alkaline to litmus with 10 per cent crystalline sod. carbonate. (4) Filter. (5) Sterilize.<sup>2</sup> (6) Prepare :—Sugar 1; litmus sol. (**S7:789**) 5; peptone water<sup>3</sup> 100. (7) Dissolve the sugar with gentle warming.<sup>4</sup> (8) Filter into T. T. (9) Sterilize 3 d. at 100C. (10) Allow the temperature on the third day just to reach 120C. and then extinguish the flame.

**Notes.**—<sup>1</sup>The usual preparation for fermentation tests of organisms. <sup>2</sup>Once dissolved and filtered peptone can be heated to higher temperature than 100C. without showing burning. <sup>3</sup>The peptone water may be replaced with advantage by nutrient bouillon in this medium. <sup>4</sup>Sugars should be dissolved at temperatures not exceeding 100C.

**M4:512.**—(1) Prepare : Sugar 10; lemco-bouillon<sup>1</sup> (**M2:113**) 1000 neutral to phenolphthalein.

**Notes.**—<sup>1</sup>Lemco bouillon is sugar-free.

**M4:513.**—(1) Prepare : Sod. chloride 5; peptone 10; water 1000; steam to dissolve. (3) Filter. (4) Add freshly prepared 1 per cent neutral red 2·5. (5) Adjust the reaction with 1 per cent sod. hydroxide to a point at which the neutral red is just decolourized. (6) Sterilize in autoclave. (7) Prepare :—10 per cent sugar in quantity to give a 1 per cent sol. when added to the neutral red peptone sol. and sterilize 10 min. at 105C. (8) Add this quantity of sugar sol. to the requisite amount of neutral red peptone sol. (9) Distribute in T. T. containing fermentation tubes. (10) Sterilize 10 min. at 105C. or 20 min. at 100C. 2 d. (11) Test the sugar by inoculation of 2 T. T. containing it, one with an organism known to ferment it and another with an organism known not to ferment it. (12) Incubate for 7 d. before making the final reading.

**M4-514.**<sup>1</sup>—(1) Use a 3 per cent agar 0.7 per cent acid to phenol phthalein. (2) Colour with litmus sol. to a purple violet colour. (3) Add 1 per cent lactose and 0.1 per cent glucose. (4) Sterilize 2 *d.* at 100°C. (5) Slope. (6) Sow by smearing the surface and stabbing into the butt.

**Notes.**—<sup>1</sup>Double sugar medium. *B. typhosus* shows a delicate growth on a violet slope and pink in the butt. *B. paratyphosus* A and B show a growth on a violet slope and pink in the butt, with gas bubbles. *B. coli* shows a growth on a pink slope and pink in the butt, with gas bubbles.

**M4-515.**—(1) Prepare: Sugar 10; peptone 20; D. W. 850. (2) Steam 20 min. (3) Add litmus sol. (**S7-787**) 50. (4) Add cautiously N-1 sol. hydroxide, if the reaction shows acid, until the neutral tint returns. (5) Distribute in quantities of 5 to 10 c.c. into T. T. containing inverted fermentation tubes. (6) Sterilize for not more than 20 min. on each of three successive days.

**M4-516.**<sup>1</sup>—(1) Prepare:—Lactose 20; glucose 0.1; dissol. phosphate 0.5; ammon. sulphate 1; sod. citrate 2; sod. chloride 5; peptone 0.05; azolitmin 0.05; D. W. 1000.

**Notes.**—<sup>1</sup>A substitute for litmus milk. A double sugar medium.

**M4-517.**<sup>1</sup>—(1) Prepare:—Peptone 20; 5 per cent pot. hydroxide 10; gelatin 75; T. W. 1000. (2) Steam 45 min. (3) Filter. (4) Add 20 per cent sugar 5 per cent, and 1 per cent neutral red 0.5 per cent. (5) Sterilize 10 min. 3 *d.* at 100°C.

**Notes.**—<sup>1</sup>For differentiation *B. coli* group of organisms in water examination.

**M4-518.**—(1) Prepare:—Bile salt 5; peptone 20; T. W. 1000. (2) Steam 2 hr. (3) Filter. (4) Add 20 per cent sugar 5 per cent and 1 per cent neutral red 0.5 per cent. (5) Sterilize 10 min. 3 *d.* at 100°C.

**M4-519.**—(1) Prepare: Nutrient agar medium containing 1 per cent sugar. (2) Adjust reaction with brom. thymol blue indicator to a pH value 6.8 to 7.2. (3) Add as indicator 0.001 per cent brom. cresol purple, or brom. cresol purple and cresol red each 0.0005 per cent, or China blue 0.0025 per cent, or China blue 0.0025 per cent with sod. rosolate 0.005 per cent.

**Notes.**—<sup>1</sup>Keep the indicator in concentrated alc. stock sol. of such a strength that a definite amount can be measured out per litre of medium, e.g. 0.1 c.c. of 1 per cent brom. cresol purple per litre of medium.

**M45-2 GLUCOSE AND MALTOSE: M4-521.**—(1) Prepare No. 1 sol.:—Peptone 5 grm.; sod. chloride 2.5 grm.; D. W. 480 c.c. (2) Sterilize in autoclave. (3) Filter. (4) Adjust reaction if necessary. (5) Prepare No. 2 sol.:—Glucose 5 grm.; litmus sol. (**S7-787**) 20 c.c.

(6) Add No. 1 sol. to No. 2 sol. (7) Filter through paper. (8) Steam 10 min. (9) Sterilize by filtration through a candle into a flask with side tubulure. (10) Distribute into T. T. from the flasks. (11) Test sterility before use.

**Notes.**—<sup>1</sup>Or other sugar.

**M4-522.**<sup>1</sup>—(1) Prepare:—Granulated peptone<sup>2</sup> 10; commercial glucose<sup>3</sup> 10; agar 18; water 1000. (2) Raise slowly in the autoclave to a temperature of 120°C. and then allow to fall to 100°C. (3) Shake, to mix, at a temperature of 100°C. (4) Have in readiness 2 one-litre flasks with funnels and thick, filter paper. (5) Place these flasks in the hot autoclave. (6) Filter. (7) Keep the unfiltered medium hot. (8) Replace the funnels and filter paper by new funnels with filter paper as soon as filtration<sup>4</sup> becomes slow. (8) Repeat the procedure as often as is necessary. (9) Distribute the filtrate into T. T.<sup>5</sup>

**Notes.**—<sup>1</sup>No adjustment of reaction of this medium is required. For cultivation of *Tineæ*, moulds, *Sporothrix*, etc. <sup>2</sup>Of Chassaing. Use any peptone which is available. <sup>3</sup>Chanut. Use any glucose which is available. <sup>4</sup>The filtration of this medium is particularly difficult and slow. <sup>5</sup>The tubes should not be capped during incubation (which may be long) but may be placed in a covered receptacle with its cover only just open.

**M4-523.**—(1) Prepare:—Peptone<sup>1</sup> Chassaing 10; D. W. 500. (2) Prepare:—French<sup>2</sup> commercial maltose 40; agar 18; water 500; peptone water 500. (3) Clear and filter.<sup>3</sup> (4) Distribute into T. T. or flasks. (5) Sterilize.

**Notes.**—<sup>1</sup>Any peptone may be used. The peptone should be brought into suspension with a little of the D. W. before being added to the bulk of the water. Solution should be effected by gentle heating. <sup>2</sup>Or ordinary maltose if not available. The maltose should be completely dissolved first in water with only gentle heat. Add to the peptone water. Dissolve the agar in the sugar peptone water. <sup>3</sup>The filtration is slow and tedious, *vide* procedure **M4-522**.

**M4-524.**<sup>1</sup>—(1) Mince finely ox liver. (2) Add 500 grm. to 1000 c.c. D. W. (3) Heat the mixture 20 min. at a temperature not exceeding 50°C. (4) Skim off fat floating on the surface. (5) Raise the temperature to boiling point. (6) Boil 10 min. (7) Pour the mixture on to a wet, thick, clean cloth. (8) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (9) Filter the fluid collected through well-wetted, thick, filter paper. (10) Add to the filtrate, peptone 10 grm. (11) Bring the vol. up to 1000 c.c. by the addition of water. (12) Adjust the reaction.<sup>1</sup> (13) Steam 30 min. (14) Filter, while hot, through well-wetted, thick, filter paper.

(15) Add to the filtrate. — Glucose 10 gram, dipot. phosphate 1 gram.

(16) Add, if required, 1 c.c. sterile defibrinated rabbit's blood to each 10 c.c. medium.

**Notes.**—<sup>1</sup>The medium solidified with agar adjusted to 4 per cent acid to phenolphthalein is suitable for the cultivation of *B. bifidus* and to a reaction of 4 per cent acid to phenolphthalein for the cultivation of *B. acidophilus*.

**M4·525.**—(1) Prepare:—Maltose 1; woodash 5; agar 10; D. W. 1000.

**M4·526.**<sup>1</sup>—(1) Prepare:—Glucose 20; sod. sulphindigotate 1; sod. formate 4; nutrient agar 1000.

**Notes.**—<sup>1</sup>For anaerobic culture.

**M4·527.**<sup>1</sup>—(1) Prepare:—Glucose<sup>2</sup> 1; casein digest medium (**M4·731**) 100. (2) Distribute to a depth of 1 in. in small T. T. containing small fermentation tubes. (3) Cover with 1·4 in. liquid paraffin. (4) Steam 90 min. (5) Add before use 3 drops alkaline egg<sup>3</sup> medium (**M2·822**). (6) Place the T. T. 20 min. in boiling water before use to expel free air. (7) Sow by means of a capillary pipette, passed through the paraffin layer, with the liquid portion of vigorously growing meat medium (**M3·51**) cultures.

**Notes.**—<sup>1</sup>For the determination of the sugar reactions of anaerobic organisms. <sup>2</sup>Or other sugar. <sup>3</sup>Used as the indicator of development of activity; for the albumin is precipitated.

**M4·528.**—(1) Prepare:—Glucose 1; bouillon<sup>1</sup> 100.

**Notes.**—<sup>1</sup>Or nutrient agar for solid medium.

**M4·53 LACTOSE: M4·531.**—(1) Prepare:—Lactose 1; bouillon<sup>1</sup> 100.

**Notes.**—<sup>1</sup>Or nutrient agar for solid medium.

**M4·54.—SACCHAROSE.**

**M4·55 MANNITE: M4·551.**—(1) Prepare:—Mannite 1; peptone 20; water 1000. (2) Make neutral to litmus. (3) Filter. (4) Add litmus sol. 47·5. (5) Distribute in T. T. (6) Sterilize.

**M4·552.**—(1) Prepare:—Mannite 1; bouillon<sup>1</sup> 100.

**Notes.**—<sup>1</sup>Or nutrient agar for solid medium.

**M4·56 GLYCERIN** *vide* **M3·1.**

**M4·57 STARCH, DEXTRIN, INULIN: M4·571.**—(1) Macerate 500 gm. finely minced beef in 1500 c.c. water in the ice chest overnight. (2) Filter through doubled gauze. (3) Bring the filtrate to the boil and add agar 1·5 per cent. (4) Boil 25 min. (5) Estimate and adjust

reaction to 0·5 per cent acid to phenolphthalein. (6) Clarify and filter. (7) Add 1 per cent starch. (8) Steam 15 min., shaking 3 times during this period to distribute the starch. (9) Distribute into T. T. (10) Sterilize 15 min. at 10 lb.

**Notes.**—<sup>1</sup>There should be abundance of water of condensation developed, and the agar must not exceed 1·5 per cent. Used for cultivation of gonococcus. For cultivation of meningococcus add 1 per cent glucose and colour the medium with litmus sol. The meningococcus acidifies the glucose while *M. catarrhalis* does not.

**M4·572.**—(1) Prepare :—Water soluble starch 2 : bouillon or nutrient agar 1000.

**M4·573.**<sup>1</sup>—(1) Prepare :—Starch 10 : meat extract (**M3·61**) 1000, 0·4 per cent to phenolphthalein.

**Notes.**—<sup>1</sup>For cultivation of gonococcus.

**M4·574.**<sup>1</sup>—(1) Prepare :—Peptone 20 ; sod. bicarbonate 1·5 ; agar 30 ; water 1000. (2) Filter, while hot, through well-wetted, thick, filter paper. (3) Make a suspension of potato starch 10 with a little of the hot filtrate and add to the bulk of the filtrate. (4) Mix. (5) Sterilize at 100C. 3 d. (6) Add, with sterile precautions, sterile litmus sol. (**S7·783**) to the hot melted starch agar to give the necessary colour.

**Notes.**—<sup>1</sup>For differentiation of *V. cholerae*.

**M4·575.**<sup>1</sup>—(1) Make a paste or suspension :—Corn flour starch 10 ; meat extract (**M3·61**) 200, with heat. (2) Heat 20 min. at 20 lb. pressure. (3) Prepare :—Starch suspension 200 ; peptone 10 ; sod. chloride 5 ; meat extract (**M3·61**) 800. (4) Steam 15 min. (5) Make the reaction 0·2 per cent acid to phenolphthalein. (6) Steam 30 min. (7) Filter, while hot, through well-wetted, thick, filter paper. (8) Add sat. litmus sol. to make a deep blue colour. (9) Distribute into T. T. (10) Sterilize 20 min. at 100C. 3 d.

**Notes.**—<sup>1</sup>For cultivation of meningococcus.

**M4·576.**—(1) Prepare a 10 per cent sol. of inulin. (2) Sterilize 15 min. at 15 lb. (3) Prepare :—10 per cent inulin sol. 1 ; serum water (**M2·311**) 10. (4) Sterilize 20 min. at 100C. 3 d.

**Notes.**—<sup>1</sup>For differentiation of pneumococci from streptococci.

**M4·58 MALT EXTRACT AND BEER WORT : M4·581.**<sup>1</sup>—(1) Prepare :—Crushed malt 1 : D. W. at 70C. 1. (2) Keep 60 min. at 60C. in a flask closed with a rubber cork. (3) Strain through muslin. (4) Steam 30 min. (5) Filter. (6) Sterilize 20 min. at 100 C. 3 d.

**Notes.**—<sup>1</sup>For cultivation of yeasts, moulds, trichophyta, etc.

**M4.582.**—(1) Prepare: Malt extract 25: water 1000.

**M4.583.**<sup>1</sup>—(1) Prepare: Agar 1.5: beer wort diluted to S. G. 1.000. 100.

**Note.**—<sup>1</sup>For cultivation of trichophyta, moulds, yeasts, etc.

**M4.584.**—(1) Prepare. Malt 1: water 10.

**M4.6 SUGAR FREE: M4.61.**—(1) Sow bouillon with *B. coli*. (2) Incubate 24 hr. (3) Boil 20 min. (4) Make a paste or suspension of about 15 gm. purified tale with a little of the dead bouillon culture. (5) Add the suspension to the culture. (6) Filter and re-filter through filter paper till clear. (7) Distribute into T. T. (8) Sterilize.

**M4.62.**—(1) Use a peptone medium (**M4.11**, etc.) or serum water (**M2.311**) or lemco bouillon (**M4.512**).

**M4.63.**—(1) Prepare bouillon 1 per cent acid to phenolphthalein from ox heart which has been "hung" 2 d. (2) Sow with *B. lactis aerogenes*. (3) Incubate 48 hr. (4) Steam 20 min. (5) Make reaction 1 per cent acid to phenolphthalein. (6) Sow with *B. coli*. (7) Incubate 48 hr. (8) Steam 20 min. (9) Test for absence of sugar by cultivation of *B. coli* or *B. lactis aerogenes*, using fermentation tubes. (10) Leave in a cool place for the growth to sediment. (11) Filter the S. N. F. through thick, filter paper or through a porcelain candle. (12) Distribute into T. T. (13) Sterilize.

**M4.64.**—(1) Sow meat extract (**M3.61**) with *B. coli*. (2) Incubate 24 hr. (3) Boil 20 min. (4) Filter. (5) Add peptone 2 per cent and sod. chloride 0.5 per cent. (6) Make neutral to phenolphthalein. (7) Test<sup>1</sup> for freedom from sugar.

**Note.**—<sup>1</sup>Sow with *B. coli*, incubate, and observe absence of gas formation.

**M4.65.**—(1) Mince finely fat-free veal. (2) Add 500 gm. to 1000 c.c. T. W. (3) Add an 18 hr. culture of *B. coli*. (4) Incubate 20 hr. at 22 C. (5) Heat the mixture 2 hr. at a temperature not exceeding 50 C. (6) Skim off fat on the surface. (7) Raise to boiling point. (8) Boil 10 min. (9) Pour the mixture on to a wet, thick, clean cloth. (10) Collect the fluid which drains through the cloth together with that obtained by squeezing the meat in the cloth. (11) Filter the fluid collected through well-wetted, thick, filter paper. (12) Add to the filtrate: Peptone 20 gm.: sod. chloride 5 gm. (13) Bring the vol. up to 1000 c.c. (14) Make the reaction 1.2 per cent acid to phenolphthalein. (15) Steam 30 min. (16) Filter while hot, through well-wetted cloth, filter paper. (17) Distribute the filtrate into T. T. or flasks. (18) Sterilize.

**M4.7 TRYPSIN DIGEST : M47.1 MEAT.<sup>1</sup>**

**Note.**—<sup>1</sup>Includes flesh of all kinds, ox, horse, sheep, or goat flesh, heart, placenta, etc.

**M4.711.**—Mince finely an average sized ox heart. (2) Add to 400 c.c. T. W. (3) Heat slowly to 75C. (4) Allow to cool to 45C. (5) Add trypsin sol.<sup>1</sup> to 1 per cent. (6) Place in incubator 2½ hr. (7) Test for peptone by the biuret test.<sup>2</sup> (8) Make faintly acid to litmus with 4 per cent acetic acid. (9) Boil 15 min. (10) Allow the solid matter to settle.<sup>3</sup> (11) Pour off the S. N. F. (12) Add :—Sod. chloride 1 gm. ; calc. chloride 0.5 gm. (13) Make faintly alkaline to litmus. (14) Steam 45 min. (15) Bring up to original vol. (16) Adjust the reaction (17) Steam 30 min. (18) Clarify and filter (**M5.1**). (19) Distribute <sup>4</sup> into T. T. (20) Sterilize.

**Notes.**—<sup>1</sup>*e.g.*, Liq. trypsin Co. (A and H). <sup>2</sup>Add 1 c.c. 5 per cent copper sulphate to 5 c.c. trypsin digest, followed by 5 c.c. N.1 pot. hydroxide. Note the colour change. If the colour is pink, peptonization is complete, if bluish purple incomplete. <sup>3</sup>Or simply strain through cloth. <sup>4</sup>The medium may be solidified in the ordinary way with agar. Add, if desired, to the agar filtrate at 50C. before distributing it into T.T., 5 per cent sterile pea extract (**M 4.8221**) and 25 per cent sterile rabbit or horse serum. With this addition the medium becomes suitable for the growth of organisms such as the meningococcus.

**M4.712.**—(1) Mince finely ox heart or human placenta. (2) Add 500 gm. to 1000 c.c. T. W. (3) Make faintly alkaline to litmus. (4) heat slowly to 75C. and maintain at this temperature 10 min. (5) Cool to 37C. (6) Add pancreatic extract (**T7.1**) to 1 or 2 per cent. (7) Keep at 37C. 4 hr.<sup>1</sup> (8) Control the progress of digestion by biuret<sup>2</sup> and tryptophane<sup>3</sup> tests. (9) Make faintly acid to litmus with glacial acetic acid. (10) Raise slowly to the boiling point. (11) Boil gently 15 min. (12) Filter. (13) Adjust the reaction. (14) Add 0.2 per cent di-pot. phosphate. (15) Steam 20 min. (16) Clarify and filter. (17) Sterilize.

**Notes.**—<sup>1</sup>If the digestion is extended to 6 hr. or longer it is necessary to add chloroform or toluene. <sup>2</sup>Add 0.1 c.c., 5 per cent copper sulphate to 5 c.c. filtered digest and 5 c.c. N.1 sod. hydroxide. A pink colour indicates complete peptonization. <sup>3</sup>Add slowly bromine water to 10 c.c. neutralized and filtered digest till the maximum purple colouration is obtained. *Vide* also **M3.9232**.

**M4.713.**—(1) Mince finely fat-free horse heart. (2) Add 500 gm. to 1000 c.c. water. (3) Make faintly alkaline to litmus. (4) Heat slowly to 75C. (5) Allow to cool to 45C. (6) Add trypsin sol. to 1 per cent and 35 c.c. chloroform. (7) Place in a loose stoppered bottle. (8) Keep<sup>1</sup> 10 d. at 37C. with daily shaking. (9) Add at the end of the time trypsin sol. again to 1 per cent. (10) Keep<sup>1</sup> a further period of

10 d. at 37°C without shaking. (11) Make faintly acid to litmus with 4 per cent acetic acid. (12) Boil 15 min. (13) Allow the solid matter to settle by placing in the ice chest overnight. (14) Pour off the S. N. F. or filter through well-wetted, thick, filter paper. (15) Make the react on 12 per cent acid to phenolphthalein. (16) Steam 15 min. (17) Filter. (18) Sterilize.

**Notes.**—<sup>1</sup>The reaction must be frequently tested and made faintly alkaline to litmus.

**M4-72 PUTRESCED MEAT : M4-721<sup>1</sup>** (1) Mince finely fat-free beef or rabbit flesh. (2) Add 500 gm. to 1000 c.c. water containing 5 gm. sod. carbonate. (3) Place in incubator 20 hr. (4) Make faintly alkaline to litmus. (5) Add trypsin sol. to 2 per cent. (6) Place in the incubator for a further 20 hr. (7) Filter through well-wetted, thick, filter paper. (8) Make faintly acid to litmus. (9) Steam or boil 15 min. (10) Filter through well-wetted, thick, filter paper. (11) Bring the vol. up to 1000 c.c. (12) Make neutral to phenolphthalein. (13) Steam 30 min. (14) Filter, while hot-through well, wetted, thick, filter paper.<sup>1</sup> (15) Distribute the filtrate into T. T. (16) Add just before use 1-16th part of fresh sterile rabbit kidney. (17) Use as soon as possible after the addition of the kidney.

**Notes.**—<sup>1</sup>For cultivation of anaerobic organisms. <sup>2</sup>Occasionally it may be necessary to filter through a Doulton candle.

**M4-722.<sup>1</sup>** (1) Mince finely fat-free lean meat. (2) Add 500 gm. to 1000 c.c. T. W. (3) Heat the mixture 20 min. at a temperature not exceeding 50°C. (4) Skim off fat floating on the surface. (5) Raise the temperature rapidly to boiling point. (6) Boil 10 min. (7) Make faintly alkaline to litmus. (8) Add trypsin sol. to 2 per cent. (9) Place in incubator in an open vessel 4 d. (10) Filter through well-wetted, thick, filter paper. (11) Bring the vol. to 1000 c.c. by the addition of water. (12) Make neutral to phenolphthalein at R. T. (13) Sterilize by filtration through a Berkefeld and a Doulton candle in series. (14) Store<sup>2</sup> the medium in sterile flasks under a layer of paraffin to which sod. formate has been added to the extent of 1 per cent of the total vol. of the medium. (15) Test the sterility of the medium before use by anaerobic culture.<sup>3</sup> (16) Add before use 1-16th part of fresh sterile rabbit kidney<sup>4</sup> to 5 c.c. medium. (17) Use as soon as possible after the addition of the kidney.

**Notes.**—<sup>1</sup>A medium which inhibits the growth of *B. sporans*, *B. cereus*, and *B. tetani*, atoxic round and opening bacilli, and certain oval and rod shaped forms. <sup>2</sup>It keep about 3 weeks. <sup>3</sup>Incubate at least 7 d. <sup>4</sup>With kidney added, the medium is anaerobic.

**M4·73 CASEIN : M4·731.**—(1) Add gradually 100 gm. commercial casein to 500 c.c. boiling water containing 0·8 per cent anhydrous sod. carbonate.<sup>1</sup> (2) Raise the temperature to boiling point. (3) Add 500 c.c. cold water. (4) Make the reaction faintly alkaline to litmus. (5) Allow to cool to 45°C. (6) Add 400 c.c. pancreatic extract (**T5·1**) to the mixture. (7) Keep 4 hr. at 37°C. (8) Raise the temperature to boiling point. (9) Pour the digest mixture on to a wet, thick, clean cloth. (10) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (11) Filter the fluid collected through well-wetted, thick, filter paper. (12) Bring the vol. up to 1000 c.c. by the addition of water. (13) Add 2·5 gm. sod. chloride and 0·125 gm. calc. chloride. (14) Adjust the reaction. (15) Steam 30 min. (16) Filter, while hot, through well-wetted, thick, filter paper. (17) Sterilize in autoclave. Add to 30 c.c. sterilized casein digest in large T. T. 4 gm. desiccated agar. (19) Sterilize in autoclave. (20) Add the sterilized solution of agar in quantities of 30 c.c. to casein digest already sterilized in quantities of 70 c.c. in round quart bottles. (21) Sterilize in autoclave. (22) Make a roll culture.<sup>2</sup>

**Notes.**—<sup>1</sup>Or double the quantity of washing soda. <sup>2</sup>The preparation of rolled culture in bottles is of special service for large scale preparation of vaccines.

**M4·732.**—(1) Prepare as a suspension in a well stoppered bottle:—casein 1 ; D. W. 10. (2) Shake well to break up clumps. (3) Adjust the reaction if necessary with the help of cresol red<sup>1</sup> (**S7·781**) and phenolphthalein (**S7·771**) indicator solution. (4) Add per litre:—Pancreatic extract (**T5·1**) 60 c.c. ; toluol 5 c.c. (5) Shake to mix. (6) Digest at 39 C. 10 d., with daily shaking and addition of more toluol if necessary. (7) Add per litre, 100 c.c. 7·5 per cent hydrochloric acid. (8) Steam 20 min. (9) Filter through well-wetted, thick, filter paper. (10) Make the reaction nearly neutral to litmus with 5 per cent sod. hydroxide. (11) Preserve as stock tryptic broth or stock “tryptamine.” (12) Dilute for use — “tryptamine” 1 ; water 2 = tryptamine bouillon.

**Notes.**—<sup>1</sup>The optimum reaction for the tryptic digestion of casein is about p H = 8·1 at which point cresol red indicator sol. gives a reddish violet colour and phenol phthalein remains colourless.

**M4·733.**—(1) Add very gradually 200 gm. casein to 1000 c.c. boiling water containing 20 gm. anhydrous sod. carbonate. (2) Allow to cool to 45 C. (3) Add pancreatin 3 gm. or pancreatic extract (**T5·1**) 50 c.c. ; chloroform 15 c.c. (4) Place in incubator 5 d., shaking vigorously each day to break up clumps. (5) Add again pancreatin 3 gm. or

pancreatic extract 50 c.c. (6) Place in incubator again for 10 d. (7) Add 400 c.c. N-1 hydrochloric acid.<sup>1</sup> (8) Steam 30 min. (9) Filter, while hot, through well-wetted, thick, filter paper. (10) Add 120 c.c. N-1 sod. hydroxide to the filtrate. (11) Adjust reaction. (12) Dilute for use 1-3 with 0.5 per cent sod. chloride. (13) Sterilize.

**Notes.**—<sup>1</sup>Or 400 c.c. pure concentrated hydrochloric acid diluted with 350 c.c. water.

**M4-734.**<sup>1</sup>—(1) Add 20 c.c. sterile human or rabbit blood to 100 c.c. D. W. in a flask furnished with a rubber cork and containing glass beads. (2) Shake vigorously to prevent formation of large fibrin masses. (3) Add:—Pancreatic extract (T5-1) 5 c.c.; enterokinase sol. (T5-5) 5 c.c. chloroform 1.5 c.c. (4) Shake to mix. (5) Incubate 8 d. with daily shaking for the first 3 d. (6) Remove the flask without disturbing the sediment. (7) Pipette off for use, with sterile precautions, as much clear S. N. F. as possible. (8) Filter the remainder through sterilized, filter paper. (9) Prepare: Sterile blood fluid digest at 45C., 1:1 melted trypt-casein agar (M4-731) at 45C., 4.

**Notes.**—<sup>1</sup>For cultivation of *B. influenzae*.

**M4-74 BLOOD: M4-741.**—(1) Obtain ox or sheep blood from the slaughterhouse. (2) Allow to clot. (3) Decant and store the serum in an ice chest. (4) Mince the clot. (5) Add 500 gm. clot to 1000 c.c. D. W. (6) Heat the mixture 20 min. at a temperature not exceeding 50C. (7) Raise slowly to boiling temperature. (8) Boil 10 min. (9) Pour the mixture on to a wet, thick, clean cloth. (10) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (11) Cool to 37C. (12) Make slightly alkaline to litmus. (13) Add pancreatic extract to 1 per cent. (14) Keep at 37C. for 6 to 48 hr. (15) Proceed further as from step (5) of M3-923.

**Notes.**—<sup>1</sup>Useful for primary isolation of delicate organisms—especially when cleared with decanted serum.

**M4-75 BLOOD SERUM: M4-751.**—(1) Prepare:—Ox or sheep serum 1; T. W. 1. (2) Boil till the mixture becomes milky. (3) Add to the mixture pancreatic extract (T5-1) to 10 per cent. (4) Digest at 60C. overnight. (5) Filter through thick, filter paper. (6) Distribute into T. T. (7) Sterilize in autoclave.

**M4-76 TRYPTOPHANE: M4-761.**<sup>1</sup>—(1) Prepare:—Tryptophan 0.3; di-pot. phosphate 5; water 1000.

**Notes.**—<sup>1</sup>Used for indol production test.

**M4:8 VEGETABLE, FRUIT GRAIN: M4:81 POTATO, CARROT, TURNIP: M4:811.**—(1) Scrub a number of potatoes of unbroken skin thoroughly with a stiff brush. (2) Cut off the outer skin. (3) Cut out of the potato with a potato borer a cylinder of about 3 to 4 in. diameter. (4) Divide the cylinder diagonally into **two** parts. (5) Wash in running water overnight. (6) Place each half cylinder<sup>1</sup> in a large T. T. or potato tube containing at the bottom thoroughly wet cotton wool. (7) Sterilize 20 min. at 120C.

**Notes.**—<sup>1</sup>To obtain glycerinated potato, soak the cut out cylinder in 6 per cent glycerin and soak the wool in the T. T. also in 6 per cent glycerin.

**M4:812.** (1) Wash and scrub the potato thoroughly. (2) Peel and remove eyes. (3) Prepare cylinders by means of an apple corer or a large cork borer. (4) Divide the cylinder diagonally into **two** parts. (5) Soak for not more than 24 hr. in 1-1000 sod. carbonate. (6) Place on top of sterilized cotton wool moistened with sterile water in the bottom of a tube. (7) Sterilize.

**M4:813.** (1) Grate finely-washed, peeled potatoes. (2) Add 1000 gm. to 1000 c.c. water. (3) Heat the mixture 20 min. to a temperature not exceeding 50C. (4) Raise to boiling temperature. (5) Boil 10 min. (6) Pour the mixture on to a clean, thick cloth. (7) Collect the fluid which drains through the cloth together with that obtained by squeezing the cloth. (8) Filter the fluid collected through thick, filter paper. (9) Add the filtrate to an equal quantity of D. W. (10) Steam 60 min. (11) Add glycerin to 4 per cent. (12) Mix well. (13) Filter. (14) Distribute into T. T. (15) Sterilize.

**M4:814.**—(1) Cover a half cylinder of potato with 6 per cent glycerin. (2) Steam 30 min. (3) Discard the fluid covering the potato. (4) Sterilize 30 min. at 100C.

**M4:815.**—(1) Soak diagonally cut cylinders of potato in 1-1000 sod. carbonate 24 hr. (2) Transfer to 5 per cent glycerin 24 hr. (3) Distribute in T. T. containing a pledget of wool at the bottom. (4) Fill up the tubes with the 5 per cent glycerin. (5) Sterilize 3 d. at 100C. (6) Pour off the glycerin at the time of use.

**M4:816.**—(1) Scrub a number of potatoes with unbroken skin thoroughly with a stiff brush. (2) Cut off the outer skin. (3) Cut into slices about 10-15 mm. thick, parallel to their long axes. (5) Dry the slices with filter paper. (6) Lay them in Petri dishes. (7) Sterilize 25 min. at 120C.

**M4-817.** (1) Peel potatoes. (2) Cut into large pieces. (3) Boil in water. (4) Pass through a grater. (5) Distribute the grated material in a layer in Petri dishes. (6) Sterilize 20 min. at 120°C.

**M4-818.** (1) Mince finely well washed carrots. (2) Press out the juice from the minced carrot. (3) Prepare: Carrot juice 4; water 1. (4) Add agar to 3 per cent. (5) Steam 45 min. (6) Clear with white of egg. (7) Adjust reaction. (8) Filter. (9) Distribute into T. T. (10) Sterilize.

**M4-819.**—(1) Grate finely-washed, peeled potatoes. (2) Add 2 parts 4 per cent glycerin to 1 part potato gratings. (3) Boil. (4) Filter. (5) Use the filtrate as potato extract.

**M4-82 PEA, BEAN: M4-821.** (1) Mince finely haricot beans. (2) Add 250 gm. to 1000 c.c. D. W. or clear T. W. (3) Heat the mixture 20 min. at a temperature not exceeding 50°C. (4) Raise the temperature to boiling point. (5) Boil 10 min. (6) Add 1 c.c. 1 per cent sod. bicarbonate. (7) Add sod. chloride 10 gm. (8) Steam 45 min. (9) Bring the vol. up to 1000 c.c. by addition of D. W. (10) Filter, while hot, through thick, filter paper. (11) Add saccharose 20 gm. (12) Distribute into T. T. (13) Sterilize.

**M4-822.**<sup>1</sup> (1) Prepare: Pea flour 50; sod. chloride 100; D. W. 1000. (2) Steam 30 min. with occasional stirring. (3) Filter through thick, filter paper. (4) Sterilize. (5) Use in 5 per cent addition to trypticar (**M4-71**).

**Note.**—<sup>1</sup>Used as an addition to nutrient medium for the cultivation of the meningo-coccus.

**M4-83 BANANA, PRUNE, RAISIN: M4-831.** (1) Mince finely bananas, prunes, or raisins. (2) Add 75 gm. to 1000 c.c. water. (3) Heat the mixture 20 min. at a temperature not exceeding 50°C. (4) Raise the temperature to boiling point. (5) Boil 10 min. (6) Strain the fluid through a thick, clean cloth and squeeze out the residue of the fluid. (7) Adjust the reaction. (8) Filter through well wetted thick, filter paper. (9) Distribute into flasks or T. T. (10) Sterilize.

**M4-832.** (1) Use banana in the form of cut cylinders or as 20 per cent pulp incorporated with nutrient agar.

**M4-84 FLOUR, BREAD: M4-841.** (1) Prepare: Wheat flour 150; mag. sulphate 0.5; pot. nitrate 1; glucose 15; water 1000.

**M4-842.** (1) Add just as much D. W. to whole bread crumbs as they will soak up. (2) Sterilize in the autoclave.

**M4·843.**—(1) Place a layer half an inch thick of stale bread crumbs on the bottom of a flask. (2) Add just as much water as they will soak up. (3) Sterilize 30 min. at 100C., 3 d.

**M4·844.**—(1) Prepare :—Grated bread 2; water 1.

**M4·845.**—(1) Soak slices of white bread in D. W. (2) Place in covered glass dishes. (3) Sterilize 20 min. at 115C.

**M4·85 YEAST : M4·851.**—(1) Prepare :—Yeast ash 10; ammon. tartrate 10; saccharose 100; D. W. 1000.

**M4·852.**—(1) Prepare :—Pressed yeast 75; D. W. 1000.

**M4·853.**—(1) Add 100 gm. yeast to 1000 c.c. water. (2) Boil 10 min. (3) Filter through well-wetted, thick, filter paper. (4) Adjust the reaction. (5) Distribute into flasks or T. T. (6) Sterilize.

**M4·854.**—(1) Keep 100 gm. yeast at 50C. 24 hr. or till liquefaction is complete by autolysis. (2) Dilute with water to 400 c.c. (3) Steam 30 min. (4) Adjust reaction. (5) Filter. (6) Make up the vol. to 1500 c.c. (7) Solidify with agar.

**M4·855.**<sup>1</sup>—(1) Prepare :—Baker's or brewer's yeast 1; water 10. (2) Heat the mixture 20 min. at a temperature not exceeding 50C. (3) Steam 2 hr. (4) Filter and refilter through well-wetted, thick, filter paper. (5) Prepare :—Yeast decoction at 15C., 2; melted 2·5 per cent<sup>2</sup> agar sol.<sup>3</sup> of reaction pH = 7·4, 3. (6) Sterilize in autoclave.

**Notes.**—<sup>1</sup>For cultivation of meningococcus. <sup>2</sup>With a semi-liquid agar (0·5 per cent) viability can be preserved with stab cultures much longer than with a stiffer agar. <sup>3</sup>With or without addition of peptone and sod. chloride.

**M4·856.**<sup>1</sup>—(1) Make a suspension :—Drained or centrifuged brewer's yeast 200 gm.; water 1000. (2) Add di-hydrogen sod. phosphate 2 gm. (3) Adjust reaction. (4) Add 5 c.c. chloroform. (5) Keep 48 hr. at 37C. with occasional shaking. (6) Distribute into T. T.

**Note.**—<sup>1</sup>Yeast autolysate.

**M4·857.**—(1) Prepare :—Baker's yeast 1; water 5. (2) Boil 20 min. with vigorous stirring. (3) Place in a tall glass vessel 24 hr. (4) Decant the S. N. F. (5) Make neutral to litmus.

**M4·858.**<sup>1</sup>—(1) Prepare :—Peptone 20; sod. chloride 5; N-1 sod. hydroxide 7; yeast extract<sup>2</sup> (**M4·857**) 1000.

**Notes.**—<sup>1</sup>For production of diphtheria toxin. <sup>2</sup>A meat extract neutralized to litmus and made from the best steak is also used.

**M4·86 HAY, STRAW : M4·861.** (1) Prepare : Dried chopped hay 10; D. W 1000, in a well-stoppered bottle. (2) Heat to 70C. (3) Close tightly with stopper. (4) Heat 3 hr. at 60C. on a water bath.

(5) Steam 60 min. (6) Filter through thick, filter paper. (7) Distribute into T. T. (8) Sterilize

**M4\*862.** (1) Mince finely hay or straw. (2) Add 15 to 20 gm. to 1000 c.c. water. (3) Heat the mixture at a temperature not exceeding 50C. (4) Raise the temperature rapidly to boiling point. (5) Boil 10 min. (6) Adjust reaction. (7) Filter. (8) Distribute into flasks or T. T. (9) Sterilize.

**M4\*863.**<sup>1</sup> (1) Prepare : -Agar 15 ; straw decoction 1000.

**Notes.**—<sup>1</sup>For cultivation of amoeba.

**M4\*87 VITAMINE, HORMONE : M4\*871.**<sup>1</sup>—(1) Prepare : Finely minced ox heart 500 ; peptone 10 ; sod. chloride 5 ; contents of one egg ; prepared agar (**M1\*11**) 16<sup>2</sup> ; water 1000. (2) Heat at a temperature not exceeding 68C. till the red colour of the mixture turns to brown. (3) Make faintly alkaline to litmus. (4) Add after this alkalization 1 c.c. N-1 sod. hydroxide per litre. (5) Steam 60 min. (6) Separate the clot formed from the sides of the vessel. (7) Steam 90 min. (8) Allow to stand at R. T. 10 min. (9) Pipette off the fluid and place in a tall cylinder. (10) Leave 20 min. (11) Skin off the fat from the surface. (12) Distribute into T. T. (13) Sterilize at 100C. 3 d.

**Notes.**—<sup>1</sup>A medium which is not filtered at any stage through any cloth, filter paper or cotton wool. If filtration is needed it should be through glass wool. <sup>2</sup>A less amount of agar, e.g., 0.5 per cent instead of 1.6 per cent, will give a medium in which, if sealed and kept in the incubator, meningococcus and gonococcus grown by the stab culture method will live for 2 or 3 m.

**M4\*872.**—(1) Extract 4 d. at 37C. with 100 c.c. 30 per cent alc. 20 gm. of either egg yolk, oats, pea flour, or acetic fluid. (2) Allow to deposit. (3) Decant the S. N. F. for use. (4) Add 0.25 per cent S. N. F. to the medium to be used.

**M5 MEDIUM CLARIFICATION : M5\*1.** (1) Beat up the white of one or two eggs<sup>1</sup> along with the crushed shells in about 20 c.c. water. (2) Add to the medium little by little before filtration and at a temperature not exceeding 60C. (3) Stir to mix. (4) Steam 30 min. (5) Remove from steamer and shake up well to mix. (6) Steam again 15 min. (7) Filter in the steamer through thick, filter paper or through two layers of absorbent cotton wool. (8) Re-filter, if necessary, the first portion of the filtrate.

**Notes.**—<sup>1</sup>Raw meat juice, 15 c.c. per litre of medium, may be substituted for 1 pt. of egg.

**M5\*2.**—(1) Wash thoroughly ordinary cheese cloth. (2) Fold to form a double thickness 12 × 12 in. (3) Place on the surface of a

roll of absorbent cotton wool,  $10 \times 10$  in. (1) Fit the whole into an 8 in. funnel, packing down towards the outlet. (5) Decant the medium to be filtered gently into this filter. (6) Pass successive portions of the first filtrate a second time through the filter until the pores are filled and the filtrate comes through clear.

**M5.3.** (1) Mix in 5 gm. of the finest possible mag. carbonate per litre of medium. (2) Steam 30 min. (3) Filter.

**M5.4.**—(1) Add immediately, before adjusting the reaction, a fragment of calc. chloride and immediately after a small piece of di-sod. phosphate. (2) Filter.

### **M6 MEDIUM COAGULATION.<sup>1</sup>**

**Notes.**—<sup>1</sup>Syn. inspissation.

**M6.1.** (1) Coagulate<sup>1</sup> the serum or other medium contained in T. T. in the sloping position in an inspissator, or over steam<sup>2</sup> at temperatures varying from 65 to 90C.<sup>3</sup> according to the degree of transparency required.<sup>4</sup>

**Notes.**—<sup>1</sup>The process may take several hours. A method of testing completeness of coagulation is to tap the T. T. and note whether the contents show a vibration; if so, the coagulation is incomplete. <sup>2</sup>As by placing at the top of a steam sterilizer or simply over boiling water. <sup>3</sup>If coagulation is effected at a temperature not exceeding 65 to 70C, the serum remains transparent. Above 70C, the serum becomes opaque. Its usefulness is not thereby greatly affected and the coagulation is more rapid. <sup>4</sup>Sterilization may be effected in the ordinary way 20 min. at 115C, after filling in the T. T. with 0.85 S.S.S. to cover the medium. The salt sol. is poured off at the time of use.

**M6.2.** (1) Fix 2 pieces of glass rod<sup>1</sup> by means of plasticine to an asbestos sheet.<sup>2</sup> (2) Place the asbestos sheet with tubes of medium in the hot air sterilizer. (3) Heat 30 min. at 80C. or until coagulation is effected.

**Notes.**—<sup>1</sup>To give the requisite slope to the T. T. containing medium. <sup>2</sup>To prevent charring of the medium.

**T7 TRYPSIN PREPARATION. T7.1.** (1) Free fresh pig<sup>1</sup> pancreas from fat as far as possible. (2) Mince finely and weigh. (3) Add 3 c.c. 0.5 per cent hydrochloric acid<sup>2</sup> for every gm. of the minced pancreas. (4) Stir the mixture at intervals for 30 min. (5) Add 6.4 c.c. 5 per cent sod. hydroxide for every 100 c.c. of 0.5 per cent hydrochloric acid used.<sup>3</sup> (6) Stir well and filter through folded filter paper.<sup>4</sup> (7) Shake up with a little toluol. (8) Make the reaction less acid by the cautious addition of 10 per cent sod. hydroxide.<sup>5</sup> (9) Store in a stoppered bottle in a cool, dark place.

**Notes.**—<sup>1</sup>Goat and sheep pancreas afford quite satisfactory trypsin and are largely used in India where the use of pig pancreas, whatever may be its advantages, would

be objectionable. <sup>2</sup>This can be prepared approximately by dissolving 13.7 c.c. pure concentrated hydrochloric acid (S.G. 1.16) to make 1000 c.c. with D. W. <sup>3</sup>The necessary reaction of about pH = 0.7 which results in a readily filterable mass. <sup>4</sup>The optimum amount of acid has been selected which the filtrate is perfectly clear. <sup>5</sup>The optimum condition for the preservation of trypsin seems to be about pH = 5.6. This can be obtained roughly by adding the alkali until a reaction of about 3 c.c. gives only a faint reddish tinge with a few drops of 0.02 per cent alc. methyl red indicator sol. (S7-792).

**T7-2.** (1) Free fresh pig pancreas from fat as far as possible. (2) Mince finely and weigh. (3) Add 3 times its weight of D. W. and its own weight of strong alc. (4) Shake well in a large bottle. (5) Allow to stand 3 d. at R. T. shaking occasionally. (6) Strain through muslin and filter through a folded filter paper. (7) Measure the filtrate.<sup>1</sup> (8) Add 1 c.c. concentrated hydrochloric acid<sup>2</sup> for each litre. (9) Keep for a few days and when the precipitate which forms has settled filter.<sup>3</sup>

**Notes.**—<sup>1</sup>Comes through very slowly. <sup>2</sup>If required immediately there is no need to add the hydrochloric acid, the function of which is to retard the slow auto-destruction of the trypsin. <sup>3</sup>The filtrate keeps indefinitely, in a well-stoppered bottle.

**T7-3.**—(1) Mince finely fat-free fresh pig pancreas. (2) Add it to 9 times its weight of D. W. (3) Extract under chloroform 24 hr. with occasional shaking. (4) Decant the fluid and add to it the fluid obtained by expression of the residue. (5) Filter through glass wool. (6) Add ether as preservative. (7) Keep at R. T. in the dark.

**T7-4.**—(1) Add to a finely chopped pig pancreas 40 c.c. glycerin and 160 c.c. D. W. (2) Extract for 3 d. in the ice chest, adding a small piece of camphor to inhibit putrefaction.

**T7-5.**<sup>1</sup> (1) Extract duodenal mucous membrane of pig, sheep, or goat in chloroform-water. (2) Add to pancreatic extract to 5 per cent for purposes of digestion.

**Notes.**—<sup>1</sup>Enterokinase.

**T7-6.**<sup>1</sup> (1) Grind up fresh pig pancreas with fine sand. (2) Prepare: Triturated pancreas 1; 20 per cent alc. 3; (3) D.W. together in a closed vessel 7 d. (4) Filter. (5) Use in mixture:—Substrate 40; sod. bicarbonate 0.1; alc. pancreatic extract 1; water 10. (6) Keep at temperature 45°C. 1 hr.

**Notes.**—<sup>1</sup>See paper of Harvey, B.P.

## INDEX.

- ACID**—WHEY M3-752, M3-753.
- B. ACIDOPHILUS**—MEDIUM M4-5241.
- B. AGNES**—MEDIUM M2-921, M2-922.
- B. AEROGENES**—MEDIUM M3-3811, M4-237, v. also COLI-AEROGENES GROUP.
- AGAR**—DRIED M1-22—FIBRE PREPARATION—M1-11—1-13 — POWDERED  
M1-21, M1-22—SOLIDIFICATION M2-1313—SOLUTION M2-1313.
- ALKALI**—BLOOD MEDIA M2-225, M2-228, M2-232—CASEIN MEDIUM M3-763  
—MEAT MEDIUM M3-51—SERUM MEDIA M2-36, M2-61.
- AMMONIA**—PRODUCTION M4-2461, M4-18.
- ANÆROBE**—MEDIA M2-812, M2-813, M3-51, M3-8111, M3-813, M3-814, M4-526,  
M4-527, M4-721, M4-722, M4-863.
- ARONSON**—MEDIUM M3-415.
- ASCITIC FLUID**—MEDIA M2-4202, M2-72, M2-922, M3-814, M4-872.
- ASPARAGIN**—MEDIA M4-211—M4-219.
- BACTERIA**—AMMONIA PRODUCTION M4-2461—NITRATE PRODUCTION  
M4-2461—NITROGEN FIXING M4-2421—SOIL M4-3101.
- BANANA**—MEDIA M4-83.
- BARSIEKOW**—MEDIUM M3-772.
- BEAN**—MEDIA M4-82.
- BEER WORT**—MEDIA M4-58.
- BEIJERINCK**—NO. 1 SOLUTION M4-412—NO. 2 SOLUTION M4-13.
- B. BIFIDUS**—MEDIUM M4-5241.
- BILE**—MEDIA M3-31.
- BILE SALT**—MEDIA M3-31, M3-341, M4-518.
- BIURET**—TEST M3-9231, M4-7112, M4-7122.
- BLOOD**—ALKALI MEDIA M2-225, M2-228, M2-232—CITRATED M2-25—DIGEST  
MEDIA M2-27, M3-93, M4-74—MEDIA M2-116, M2-2, M2-27,  
M2-6, M3-93, M3-94.
- BLOOD CULTURE**—BOUILLON M3-9221.
- BLOOD SERUM**—DIGEST MEDIA M2-62, M4-75—MEDIA M2-36 M2-61—WATER  
M2-31.
- BOUILLON**—MEDIA M2-11.
- BRAIN**—MEDIA M3-84.
- BRILLIANT GREEN**—MEDIA M2-2462, M3-34.
- BREAD**—MEDIA M4-84.
- BROM. CRESOL PURPLE**—CRESOL RED MEDIUM M3-241, M4-519—INDICATOR  
SOLUTION M3-2411, M3-2421, M4-5191, S7-779—MEDIA  
M3-24, M4-519.
- BROTH**—MEDIUM M2-1001.

- CAFFEINE**—MEDIA M337.  
**CALCIUM CARBONATE**—MEDIA M3-27, M4-42.  
**CALCIUM CHLORIDE**—WHEY M3-754.  
**CAPALDI PROSKAUER**—MEDIUM NO. 1 M4-214—MEDIUM NO. 2 M4-551.  
**CARBOHYDRATE**—MEDIA M4-5.  
**CARBOLIC ACID**—MEDIA M3-32.  
**CARROT**—MEDIA M4-81.  
**CASEIN**—ALKALINE MEDIUM M3-763—MEDIA M3-76, M4-73.  
**M. CATARRHALIS**—MEDIUM M4-5711.  
**CHASSAING PEPTONE**—M4-14.  
**CHINA BLUE**—MEDIA M3-32, M3-722, M4-519—PHENOL RED MEDIUM M3-233—ROSOLIC ACID MEDIA M3-231, M3-232, M3-722, M4-519.  
**CHINA GREEN**—MEDIUM M3-36.  
**CHLOROFORM**—STERILIZATION M2-237, M2-45, M2-68.  
**V. CHOLERAE**—MEDIUM M2-225, M2-823, M2-824, M3-415, M4-5741.  
**CILIATES**—SOIL—M4-2451.  
**CLARIFICATION**—MEDIA M5.  
**COAGULATION**—MEDIA M6.  
**B. COLI MEDIA**—M3-4131, M4-5171.  
**COLI-AEROGENES GROUP**—MEDIA M3-381, M4-233, M4-5171.  
**CONRADI-DRIGALSKI**—MEDIUM M3-331.  
**CRESOL RED**—INDICATOR SOLUTION M3-2511, M4-7321—MEDIUM M3-244, M3-25.  
**CRYSTAL VIOLET**—MEDIUM M3-331.  
**DENITRIFYING**—ORGANISMS M4-4511, M4-4531, M4-4551, M4-4561.  
**DEXTRIN**—MEDIUM M4-57.  
**DIEUDONNE**—MEDIUM M2-225.  
**DIEUDONNE-LENTZ**—MEDIUM M2-228.  
**DORSET**—MEDIUM M2-811.  
**B. DIPHTHERIÆ**—MEDIUM M2-59, M2-61, M2-62, M2-812, M3-3941, M3-924, M4-878—TOXIN M4-8581.  
**DOUGLAS**—MEDIA M4-7.  
**DRIGALSKI-CONRADI**—MEDIUM M3-331.  
**DUNHAM**—SOLUTION M4-12.  
**B. DYSENTERIÆ**—MEDIUM M3-232.  
**EGG**—CLARIFICATION M5—MEDIA M2-8, M4-872.  
**ENDO**—MEDIA M3-412-3-411.  
**ENTERO-KINASE**—PREPARATION T7-5.  
**EOSIN**—METHYLENE BLUE, MEDIUM, M3-381.  
**ETHER**—STERILIZATION M2-241, M2-253, M2-54.  
**FAT**—MEDIA M2-9.  
**FATTY ACID**—MEDIA M2-9.  
**FERRIC TARTRATE**—MEDIA M4-431, M4-432.  
**FILTRATION**—GLASS WOOL M4-8711—MEDIA M5.  
**FISH**—MEDIUM M2-117.  
**FLAGELLATES**—SOIL M4-2451.  
**FLOUR**—MEDIA M4-81.  
**FORMALIN**—STERILIZATION M2-261, M2-57.

**FRUIT**—MEDIA M48.

**GELATIN**—MEDIA M242—WATER MEDIUM M431.

**GIBSON**—MEDIUM M4574.

**GILTAY SOLUTION** M4455.

**GLASS WOOL FILTRATION** M48711.

**GLUCOSE** BOUILLON M4528 MEDIA M232, M239, M243, M258, M261, M263, M266, M2722, M28112, M2812, M2841, M3312, M3314, M3351, M3352, M3851, M3924, M415, M416, M4218 M4234, M4235, M4236, M4244, M4411, M452, M45711.

**GLYCERIN**—MEDIA M2341, M241, M2711, M2712, M27221, M2844, M2911, M2921—2923, M31, M3313, M3316, M414, M416, M4211, M4212, M4215, M4218, M4219, M4221, M4229, M4234 4237, M456, M48111, M4813—M4815, M4819.

**GRAIN**—MEDIA M48.

**GONOCOCCUS**—MEDIA M2221, M22211, M22212, M264, M267, M3753, M3851, M4571, M4573, M4871.

**HAY**—MEDIA M486.

**HISS**—SERUM WATER M2311, M2312.

**HORMONE**—MEDIA M487.

**HYDROCELE FLUID**—MEDIUM M27111.

**HYPHOMYCETES** MEDIA M39211, M416, M417, M4581, M4583.

**SP. ICTEROHÆMORRHAGIÆ** MEDIA M246—248.

**INDICATOR**—MEDIA M32, M33, M372, M45—MILK MEDIA M372, SOLUTIONS M32, M33, M372, 8776—779, T7-1005.

**INDOL TEST**—MEDIUM M263, M4215, M4761.

**B INFLUENZÆ** MEDIA M2212, M2213, M2224, M22335, M2245, M2246, M2255, M2272, M2931, M3932, M47341.

**INSPISSATION**—MEDIA M6.

**INULIN**—MEDIUM M4576.

**IRON**—SALTS MEDIA M443.

**KUTSCHER**—MEDIUM M244

**LACTOSE**—BOUILLON M4531—MEDIA M2229, M3316, M3324, M3331, M3352, M3372, M341, M411, M4233, M4235, M4241, M453,

**LEAD**—SALTS MEDIUM M44.

**LEAD ACETATE**—MEDIUM M4441—444.

**LEISHMANIA**—MEDIUM M2216, M2221, M2226.

**LEMCO**—MEDIA M2113, M362.

**LEVINTHAL** MEDIUM M2238.

**LIPMAN AND BROWN**—MEDIUM M4411.

**LITMUS**—MEDIA M321, M3721, 6376—MILK SUBSTITUTE M4516

**LIVER**—MEDIUM M2115.

**LOCKE**—FLUID M24602.

**LORRAIN SMITH**—MEDIUM<sub>g</sub> M236.

**LÆFFLER**—MEDIUM M235.

**LUBENAU**—MEDIUM M28413

**MACCONKEY**—MEDIUM M3316.

**MALACHITE GREEN**—MEDIUM M335.

**MALARIA**—CULTIVATION M2215.

**MALT EXTRACT**—MEDIA M458,

**MALTOSE**—MEDIA M452

**MANNITE**—BOUILLON M4552 MEDIA M4210, M4231, M4242, M4413, M4415

**MARTIN**—PEPSIN DIGEST BOUILLON M3912—PEPSIN DIGEST SOLUTION M3911.

**MEAT**—ALKALI MEDIUM M351, M355, M472.

**MEDIA**: ACID DIGEST M2414 AGAR BOUILLON M243 AGAR FERRIC M411—413 AGAR GELATIN M244 AGAR POWDER M421, M422 ALKALI BLOOD M2225, M2228, M2232 ALKALI CASEIN M3763 ALKALI EGG M2822 ALKALI MEAT M351—ALKALI SERUM M239, M261—ANTHROPE M2812 M2813, M351, M3811, M3813, M3814 ARONSON M3415 ASCITIC FLUID M24262, M272, M2922, M3814, M4872 ASPARAGIN M4211—4219 BANANA M283 BARSZKOW M3772 BELJERINCK NO. 1 M4412 BELJERINCK NO. 2 M4413 BILE M331 BILE SALTS M230, M3341, M4418 BLOOD M2416, M242 BLOOD DIGEST M227, M399, M474 BLOOD PLASMA M23 BLOOD SERUM M2211, M2223, M23—26, M3303 BLOOD SERUM DIGEST M262, M475 BODY FLUID M27 BOUILLON M241 BRAIN M381 BREAD M481 BRILLIANT GREEN M2462, M331 BROM CRESOL PURPLE M326 BROTH M241 CAFFEINE M237 CALC CARBONATE M327, M427, M4421 CAPALLE PPOSKALIEB NO. 1 M4204 CARBOHYDRATE SUGAR CARBOLIC ACID M332 CARROT M481 CASEIN M376, M473 CHINA BLUE M323 CHINA BLUE PHENOL RED M223 CHINA BLUE ROSOLIC ACID M323, M3232, M3722 CHINA GREEN M3361 CITRATED BLOOD M225 CLARIFICATION M5, M2418, M2422 COAGULATION M6—COMPOSITION M24 CONRAD DRIGALSKI M3331 CRESOL RED M3241, M325 CRYSTAL VIOLET M3331—DEFIBRINATED BLOOD M222 M223 DENTIN M457 DIETDONNE M2225, M2239 DIETDONNE LENTZ M2428—DIGEST BLOOD M227, M26, M393, M394—DORSET M2811 DOUBLE SUGAR M4541 M4546 DOUGLAS M47—DRIED MILK M277—DRIGALSKI-CONRAD M3331 DUNHAM M411—ECONOMY M2462 EGG M28, EGGIN METHYLENE BLUE ORGANS TAYS M29—FATTY ACID M29—FERRIC SALT M255 M4431 M4432 FERRIC AGAR M411—413 FILTRATION M4151 M2417 FLOUR M481 FREGONI M281 FROTH M29 FUCHSIN M3411—4413—GELATIN ACID M244 GELATIN BOUILLON M242 GELATIN WATER M336 GIBSON M4571 GILLAY M4455 GLYCERIN M340, M241, M2711, M2712, M27221, M2841, M2911, M2922, M2923 M2661, M341, M3413, M3316 M4141, M415, M3411, M3412, M4215, M4218, M4219, M4221, M228, M230—500 M446, M4811, M4813—4815, M4415—4417—500 M736 M239, M243, M258, M261, M269, M272, M2723, M2812, M2812, M2941, M3312, M3314, M3401, M3402, M3841

M3-924, M4-15, M4-16, M4-218, M4-234—4-236, M4-244, M4-411,  
 M4-52, M4-5711—GRAIN M4-8—HAY M4-86—HISS M2-311,  
 M2-312—HORMONE M4-87—HYDROCELE FLUID M2-7111  
 —INDICATOR M3-2, M3-3, M4-5—INDICATOR MILK M3-72  
 —INHIBITORY SUBSTANCE M3-3, M3-4—INSPISSATION  
 M6—INSPISSATED SERUM M2-32—2-36, M2-42, M2-45, M2-51,  
 M2-53, M2-55, M2-56—INULIN M4-576—IRON SALTS M4-43  
 —KUTSCHER M2-44—LACTOSE M2-229, M3-316, M3-324, M3-331,  
 M3-352, M3-372, M3-41, M4-11, M4-233, M4-235, M4-241, M4-53—  
 LAKED BLOOD M2-24—LEAD SALTS M4-44—LEMCO M2-113,  
 M3-62—LIPMAN AND BROWN M4-411—LITMUS M3-21,  
 M3-721, M3-754—LITMUS MILK M3-721—LITMUS MILK SUB-  
 STITUTE M4-516—LIVER M2-115—LOEFFLER M2-35—  
 LORRAIN SMITH M2-63—LUBENAU M2-841—MACCON-  
 KEY M3-316—MALACHITE GREEN M3-35—MALT  
 EXTRACT M4-58—MALTOSE M4-52—MANNITE M4-214,  
 M4-2341, M4-242 M4-413, M4-55—MARTIN M3-911, M3-912—  
 MEAT M3-5 MEAT EXTRACT M3-6—MEAT EXTRACT  
 PEPTONE M2-1—MILK M2-7111, M3-7—MILK PRO-  
 DUCTS M3—N.N.N. M2-216—NASGAR M2-723—NEUTRAL  
 RED M3-261, M3-316—NITRATE M4-45—NITRIFICATION  
 M4-415, M4-416—NITROGEN FREE—NOGUCHI M3-814—  
 NOVY MACNEAL M2-226 NUTRIENT AGAR M2-13—  
 NUTROSE M2-44, M2-723, M3-331, M3-352, M3-7632, M3-771,  
 M3-772—OIL M2-91—OLEATE M2-93—OLEIC ACID M2-92  
 —OLIVE OIL M2-912—OMELIANSKY M4-221—ORGAN  
 M2-1151, M3-81, M4-711—4-713—ORGAN FRAGMENT  
 M3-81—OVARIAN FLUID M2-7111—OXALATED BLOOD  
 M2-26—PANCREATIN M2-74—PARIETTI M3-321—PAS-  
 TEUR SOLUTION M4-851—PEA M4-82—PEPSIN DIGEST  
 M3-9—PEPSIN TRYPSIN DIGEST M3-94—PEPTONE  
 M4-1, M4-521—PEPTONE DERIVATIVES M4-1—PEPTONE  
 WATER M4-12, M4-511—PETROFF M2-819—PHENOL  
 M3-32—PHOSPHATE BLOOD M2-222—PLACENTA M2-44  
 —PLEURITIC FLUID M2-7111—POTATO M2-224, M4-81—  
 POWDER AGAR M1-21, M1-22—PREPARATION M24—  
 PREPARATION AGAR M1—PROTEIN FREE M4-2, M4-4 v.  
 also SALTS ORGANIC AND INORGANIC ACIDS—  
 PROTEIN POOR—M4-31—PRUNE M4-83—RAISIN M4-83—  
 RAULIN M4-224—REACTION M2-1116, S7-76, S7-81 S7-83—7-86.—  
 ROSOLIC ACID M3-22, M3-231—ROSOLIC ACID-LACTOSE  
 M2-229—ROW M2-221—RUSSELL DOUBLE AGAR M4-514—  
 SABOURAUD M4-14, M4-522, M4-523—SACCHAROSE  
 M3-421, M4-2341, M4-54—SALTS ORGANIC ACIDS M4-4  
 v. also PROTEIN FREE MEDIA—SALTS ORGANIC ACIDS  
 M4-4 v. also PROTEIN FREE MEDIA—SELECTIVE  
 M3-3001—SERUM v. BLOOD SERUM—SERUM WATER  
 M2-31—SILICATE JELLY M4-417, M4-418—SOD. FORMATE

M4526—SOD. SALICYLATE M342—SOD. SULPHATE M341  
—SOD. TAUROCHOLATE M334—3347. M3341—SOD.  
SULPHINDIGOTATE M4526—SOIL. EXTRACT M4243—  
4245—SOPARKAR M2255—SPERM OIL. M2911—SPRONCK  
M4858—STARCH M3421. M4571—4575—STERILIZATION  
89—STRAW M486—SUGAR M2344, M2726, M327, M3362,  
M3762, M3772, M442, M4421, M45, M451, M45211, M4526  
—SUGAR FREE M21131, M21161, M4512, M46—SYNTHETIC  
M42, M44—TELLURIC ACID M3344, M336—TESTICLE  
M3851—TISSUE M381—TRYPTAMINE M4732—TRYPSIN  
DIGEST M47—TRYPTOPHANE M4761—TURNIP M481  
URINE M27111, M273—USCHINSKY M4241, M4212—  
VEGETABLE M48—VINES M4575—VITAMINE M487  
WERTHEIM M239—WHEY M375—WHITE OF EGG M283  
WHOLE BLOOD M221—WHOLE EGG M281, M282—  
WINOGRADSKY M4414, M4415, M4417—YOLK OF EGG M284.

**MENINGOCOCCUS**—MEDIA M21151, M2224, M22371, M2261, M2238, M23221,  
M252, M254, M257, M2724, M2726, M45711, M4575,  
M47114, M48221, M4855, M4871.

**METHYL RED**—INDICATOR SOLUTION 87792, T71005.

**MILK**—MEDIA M27111, M37—PRODUCTS MEDIA M37—RICE MEDIA M374.

**MOULDS**—MEDIA M34211, M416, M417, M4581, M4583.

**N. N. N.**—MEDIUM M2216.

**NASGAR**—MEDIUM M2723.

**NEUTRAL RED**—MEDIA M3261, M3316.

**NITRATE**—MEDIA M445—PRODUCTION M42461, M4444, M44491—  
REDUCTION v. DENITRIFYING.

**NITRIFYING**—ORGANISMS M44151, M44161, M44181.

**NITRITE**—PRODUCTION M42461, M44141.

**NITROGEN**—FIXING BACTERIA M42421—FREE MEDIUM M4242.

**NOGUCHI**—MEDIUM M3814.

**NOVY-MACNEAL**—MEDIUM M2226.

**NUTROSE**—MEDIA M244, M2723, M3331, M3352, M3762, M3771, M3772.

**OLEATE**—MEDIUM M293.

**OLEIC ACID**—MEDIA M292.

**OLIVE OIL**—MEDIUM M2912.

**OMELIANSKY**—MEDIUM M4221.

**ORGAN**—MEDIA M21151, M381, M4710—4713—REMOVAL M3811.

**OVARIAN FLUID**—MEDIUM M27111.

**PANCREATIN**—MEDIUM M274—PREPARATION T7.

**PARIETTI**—MEDIUM M3321.

**PASTEUR**—SOLUTION M4851.

**PEA**—FLOUR EXTRACT M4822, M4872—MEDIA M482.

**PEPSIN**—DIGEST BOTTLETON M2942—DIGEST SOLUTION M2941.

**PEPTONE**—CHASSAING M414—MEDIA M44, M4529—FALGARD M241—  
WATER M442, M4511.

**B. PERTUSSIS**—MEDIUM M22241.

**PETROFF**—MEDIUM M2819.

**PHENOL**—MEDIA M332.

**PHENOLPHTHALEIN**—INDICATOR SOLUTION M47321.

**PHENOL RED**—CHINA BLUE MEDIUM M3233.

**PHOSPHATE**—MEDIA M21117, M22224.

**PLACENTA**—MEDIUM M244.

**PLEURITIC FLUID**—MEDIUM M27111.

**PNEUMOCOCCUS**—MEDIA M2261, M45761.

**POTATO**—MEDIA M481.

**PROTEIN**—FREE MEDIUM M42 v. SALTS ORGANIC AND INORGANIC  
ACIDS—POOR MEDIA M43.

**PRUNE**—MEDIA M483.

**PUTRESCED MEAT**—MEDIA M472.

**RAISIN**—MEDIA M483.

**RAULIN**—SOLUTION M4224.

**RENNET**—WHEY M3751.

**RICE**—MILK MEDIA M374.

**RINGER**—SOLUTION M24802.

**ROSOLIC ACID**—INDICATOR SOLUTION M32211—LACTOSE MEDIUM  
M2229.—MEDIA M322, M3231, M3232.

**ROW**—MEDIUM M2221.

**RUSSELL**—DOUBLE SUGAR MEDIUM M4514.

**SABOURAUD**—MEDIA M414, M4522, M4523.

**SACCHAROSE**—MEDIA M42341, M3421, M454, M4851—SERUM v. BLOOD  
SERUM.

**SILICATE**—JELLY MEDIUM M4417, M4418.

**SODIUM FORMATE**—MEDIUM M4526.

**SODIUM SULPHINDIGOTATE**—MEDIUM M4526.

**SOD. SULPHITE**—MEDIA M341.

**SODIUM SALICYLATE**—MEDIUM M3421.

**SODIUM TAUROCHOLATE**—MEDIA M3314, M3317, M3341.

**SOIL**—BACTERIA MEDIA M4217, M4246, M431, M4411—CILIATES  
M42451—EXTRACT M42431—FLAGELLATES M42451.

**SOPARKAR**—MEDIUM M2255.

**SPEARM OIL**—MEDIUM M2911.

**SPIROCHAETE**—MEDIA M249, M251, M253, M256, M258, M38111, M38141.

**SPOROTHRIX**—MEDIUM M45221.

**SPRONCK**—MEDIUM M4858.

**STANDARD**—PEPTONE M21115.

**STARCH**—MEDIA M3421, M4571—4575.

**STERILIZATION**—CHLOROFORM M2237—ETHER M2241, M2253—FINGER  
M2211—FORMALIN M2261—MEDIA S9—SUL-  
PHURIC ACID M22462.

**STRAW**—MEDIUM M486.

**STREPTOCOCCUS**—MEDIA M3813, M45361.

**SUGAR**—FERMENTATION M45111—FREE MEDIA M21131, M21161, M4512,  
M46—MEDIA M2311, M2726, M33162, M327, M3762, M3772,  
M412, M4421, M45, M451, M45211, M4526—RUSSELL'S MEDIUM  
M4514.

- TELLURIC ACID** MEDIUM M3344, M3339.  
**TESTICLE** MEDIUM M3851.  
**B. TETANI** MEDIUM M47221.  
**TINEA** MEDIA M45221, M4581, M4583.  
**TISSUE** MEDIA M381.  
**TORULÆ** MEDIUM M34211.  
**TRICHOPHYTON** MEDIA M45221, M5481, M4583.  
**TRYPANOSOME** MEDIA M2231, M2234.  
**TRYPSIN** MEDIUM M271 PREPARATION T7.  
**TRYPTAMINE** BOUILLON M4732 STOCK M4732.  
**TRYPTOPHANE** MEDIUM M4761 TEST M39232, M47123.  
**B. TUBERCULOSIS** MEDIA M2812, M28412, M2911, M3814, M3815, M4228, M4236.  
**TURNIP** MEDIA M481.  
**TYPHO-COLI GROUP** MEDIA M2229, M33, M33531, M33711, M33721, M34, M3754, M4443, M4514.  
**TYPHOID GROUP** MEDIA M33, M33101, M33431, M34.  
**URINE**—MEDIA M27111, M273.  
**USCHINSKY**—MEDIA M4211, M4212.  
**VITAMINE** MEDIUM M487.  
**WATER** EXAMINATION MEDIUM M45171.  
**WATER GLASS** MEDIUM M44182.  
**VACCINES** LARGE SCALE PREPARATION M47312.  
**VEGETABLE**—MEDIA M48.  
**WERTHEIM** MEDIUM M239.  
**WHEY**—ACID M3752, M3753—CALC. CHLORIDE M3754—MEDIA M375—RENNET M3751.  
**WINOGRADSKY**—SILICATE JELLY M4417—SOLUTION M4414, M4415.  
**YEAST**—CULTIVATION M415, M4232, M4581, M4583—MEDIA M485.

# SOME CASES OF MYIASIS IN INDIA AND PERSIA, WITH A DESCRIPTION OF THE LARVÆ CAUSING THE LESIONS.

BY

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[Received for publication, January 3, 1921.]

## CONTENTS.

	Page
(1) INTRODUCTION .. .. .	132
(2) A SHORT NOTE ON SOME SPECIES OF FLIES INVOLVED IN MYIASIS IN THE EAST WITH ESPECIAL REFERENCE TO INDIA .. .. .	133
(3) CASES OF MYIASIS REPORTED FROM INDIA AND BURMA .. .. .	135
(4) HISTORY OF SIX CASES .. .. .	136
(5) TECHNIQUE .. .. .	139
(6) DESCRIPTION OF THE SPECIES OF FLY FOUND .. .. .	140
(A). <i>Pycnosoma dux</i> , Esch. .. .. .	140
(i) Larval stage .. .. .	140
(ii) Pupal stage .. .. .	149
(iii) Adult stage .. .. .	149
(B). <i>Lucilia serricata</i> , Mg. .. .. .	150
(i) Larval stage .. .. .	150
(ii) Pupal stage .. .. .	152
(iii) Adult stage .. .. .	153
(C). <i>Sarcophaga ruficornis</i> , F. .. .. .	153
(i) Larval stage .. .. .	153
(ii) Pupal stage .. .. .	156
(iii) Adult stage .. .. .	156
(D). <i>Wohlfahrtia</i> , sp. .. .. .	157
(i) Larval stage .. .. .	157
(ii) Pupal stage .. .. .	159
(iii) Adult stage .. .. .	159
(7) COMPARISON OF THE LARVAL AND PUPAL CHARACTERS .. .. .	159
(8) REFERENCES .. .. .	161
(9) EXPLANATION OF PLATES .. .. .	

## 1. INTRODUCTION.

DURING the last six years I have had the opportunity of studying four cases of myiasis in the North-West Frontier Province, India, and two cases in the Khorosan Province of Persia. As very little seems to have been done on the myiasis of these parts the cases seemed worthy of record.

I wish to thank Captain H. K. Rowntree, M.C., I.M.S., for permission to publish the clinical notes of case No. 2 which was under his care.

I am deeply indebted to Major E. E. Austen, D.S.O., to Dr. Guy Marshall and to Mr. F. W. Edwards, all of the Natural History Museum, South Kensington, who so kindly identified the flies for me.

## 2. A SHORT NOTE ON SOME SPECIES OF FLIES INVOLVED IN MYIASIS IN THE EAST WITH ESPECIAL PREFERENCE TO INDIA.

That myiasis is not a very uncommon disease in India is well known, but the number of cases, which have been recorded in detail, seem to be few, while those in which the actual species of fly causing the lesion has been identified seem very few indeed.

The commonest species appear to belong to one of the following genera—*Sarcophaga*, *Lucilia*, *Calliphora* or *Pycnosoma*.

(a) *Nasal myiasis*.

Theobald (1916) states that *Pycnosoma* larvae are frequently found in the nostrils of man.

Castellani and Chalmers (1919) says that 'peenash' is a word which may be used for the Indian rhinal myiasis caused by the larva of species of *Pycnosoma* Brauer and von Bergenstamm and by the larva of species of *Sarcophaga* Meigen, especially *Sarcophaga carnaria* Linnaeus 1758. 'The disease is spread throughout India and Assam.'

Austen (1910) reports that he received from Lieut.-Colonel Wyville Thompson, I.M.S., larvae from a case of nasal myiasis at Dehra Doon, which were a species of *Pycnosoma*.

Patterson (1909) reported in detail a case of nasal myiasis from Assam, which Austen (1910) thinks was probably due to a species of *Sarcophaga*, but from an examination of Patterson's drawings in his paper it appears to me that the larvae were probably *Pycnosoma* larvae rather than those of *Sarcophaga*, because (a) of the very marked 'islands' on the spinous rings of the abdomen, and the abdominal spines on the posterior parts of these segments, and (b) the three most

direction of the posterior end ; and (c) the groove in front of the edge of the posterior hollow. All these points seem much more like the *Pycnosoma* larvæ described in this paper than either the *Sarcophaga* larvæ described here or the figures of the larvæ of *Sarcophaga* sp. given by Brumpt (1913) or Castellani and Chalmers (1919).

Chetti (1910) reported three cases of nasal myiasis from Burma and for the same reasons his drawings may possibly represent a *Pycnosoma* larva.

Rieley and Howlett (1914) noted ten cases of nasal myiasis from Behar due to *Pycnosoma flaviceps* (?).

Treston (1916) recorded a case from Kohat, North-West Frontier Province, due to *Chrysomyia macellaria*. I was fortunate enough to have an opportunity of examining these larvæ and the flies which hatched out from them and they proved to be *Pycnosoma dux*.

Case No. 1 reported below from Kohat, North-West Frontier Province, was due to *P. dux*.

From an examination of these records it will be seen that in all these cases when the fly causing the lesions was definitely identified it proved to belong to the genus *Pycnosoma*.

The genus *Pycnosoma* is the Oriental representative of the American genus *Chrysomyia* which is the commonest cause of nasal myiasis in the Western Hemisphere.\*

#### (b) *Oral myiasis.*

Sturges (1917) reports *Chrysomyia* (*Pycnosoma*) *flaviceps* Mg. causing myiasis in the mouth of a dog in Ceylon.

Case No. 2 in the series now reported was due to *Pycnosoma* sp. from Kohat, North-West Frontier Province.

#### (c) *Cutaneous myiasis.*

The larvæ of various species of the genera *Sarcophaga*, *Lucilia*, *Calliphora*, *Pycnosoma*, and *Wohlfahrtia* have been reported from many parts of the world as causes of cutaneous myiasis in man and animals.

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\* Since writing the above Major Austen has informed me that the latest researches in the nomenclature of these genera shows that *Chrysomyia* is the proper generic name of the Oriental species, not *Pycnosoma*, while that of the American genus is *Cochliomyia*, not *Chrysomyia*.

Patton and Cragg (1913) state that 'in Gujarat it is not uncommon to see large sores full of the larvæ of *Sarcophagæ*.'

Castellani and Chalmers (1919) say that *Sarcophaga* larvæ are not uncommon in ulcers in the tropics.

Austen (1910) mentions that in India, according to Moniez (Triate de Parasitologie, p. 600) quoting Raillet, English medical men have reported very serious cutaneous myiasis due to *S. ruficornis*.\*

Case No. 4 recorded below occurred in a dog at Bannu, North-West Frontier Province, India, and was due to *S. ruficornis*, while case No. 6 in a camel in N. E. Persia was due to *Sarcophaga* sp.

With regard to the genus *Lucilia*, Patton and Cragg (1913) say that in South India *L. serenissima* Fabr. will oviposit in the sores of sickly cattle.

Castellani and Chalmers (1919) state that flies of this genus, *i.e.*, *L. caesar* and *L. sericata* deposit their eggs in ulcers, and Brumpt (1913) also says that the larvæ of *L. sericata* are often found in the ulcers of man and animals in the tropics.

Case No. 3 at Parachinar, North-West Frontier Province, India, in a woman was due to *L. sericata*.

Flies of the genus *Pycnosoma* are mentioned by Brumpt (1913) and by Roubaud (1915) as causes of cutaneous myiasis in Africa. While Joyeux (1916) reports seven cases of animal myiasis in French Guinea due to *P. bezzi*ana, Vill. (*megacephala*, Bezzi nec Fabricius).\*

Bishopp (1915) records cases in sheep in Hawaii due to *P. dux* and in Australia due to *P. rufifacies*.

Case No. 4 now recorded from Bannu, North-West Frontier Province, India, was also infected with *P. dux*.

Austen (1910) states that *Wohlfahrtia magnifica*, Schm. is responsible every year for very many cases of myiasis in man and domestic animals in South Russia, and Roubaud (1915) mentions this species in Africa.

Case No. 5 was a camel in N. E. Persia infected with the larvæ of *Wohlfahrtia* sp.

### 3. CASES OF MYIASIS REPORTED FROM INDIA AND BURMA

Lahoty (1856) reported that during nine years at Allahabad he saw 91 cases of 'peemash,' of which only two died.

\* Patton has described a large number of cases of cutaneous myiasis in man and animal due to *Chrysomya bezziana* in South India (Ann. Ent. Soc. Med. Res., Vol. VIII, No. 1, July, 1920, pp. 17-29).

Powell (1901) saw in Bombay (?) five cases of 'screw-worm disease' affecting the nose, of which three died and one was not traced. In the cases that died the maxillary antrum was also affected.

In Assam Patterson (1909) saw two fatal cases of nasal myiasis from the Tezpur district and a case which recovered from Cachar. He also saw a case of aural myiasis which died and two cutaneous cases which recovered in the Tezpur district.

Cameron (1909) reported two nasal cases with recovery, one from Jhelum and one from Peshawar.

In Burma Chetti (1910) saw three nasal cases, one of which died, and Keelan (1910) saw a nasal case at Pusa which recovered.

Austen (1910) records Thompson's nasal case at Dehra Doon which got better.

Only one death occurred among the ten cases of nasal myiasis recorded in Behar by Rieley and Howlett (1914).

At Kohat, North-West Frontier Province, Treston (1916) had a nasal case which did not prove fatal and case No. 1, which was from the same place, also recovered.

In these collected cases, there are 117 of the nasal variety of which only 9 died, *i.e.* a mortality of 7.7 per cent, while Manson (1917) says that out of 13 cases of the American form of nasal myiasis collected by Laboulbène 9 died and out of 31 cases collected by Maillard 21 died, *i.e.* a mortality of over 44 per cent on the 41 cases.

From these figures it would appear that the type of nasal myiasis found in America is much more severe than that found in India.

#### 4. HISTORY OF SIX CASES.

##### *Case No. 1—(Nasal myiasis in man).*

20-8-14. A mule driver, aged 20, at Kohat, North-West Frontier Province, India. This patient had had a fracture of the nasal bone and about 6 weeks later came to hospital again stating that 'worms were coming out of his nose.' He complained of a burning pain in his nose with very acute exacerbations. When examined in one of these attacks he was seen to writhe on the bed in agony and to cry out. Afterwards he would say that 'it felt as if something was eating the inside of his nose.'

He had a very severe headache; his face was swollen over the cheeks and nose and there was a continuous sero-sanguineous discharge from

both nostrils, smelling like putrid meat. His voice was husky and nasal and he could only swallow with difficulty.

On nasal examination the turbinates were so deeply swollen and congested that the nasal passages were occluded and nothing else could be made out. The back of the throat and pharynx was congested and a sanguineous discharge was coming from the posterior nares. The roof of the mouth at the junction of the hard and soft palates was inflamed. The breath was very foul.

The bowels were constipated and the temperature was 101.4°F.

The nose was syringed out with carbolic lotion, but no larvæ came away. The syringing caused an acute attack of pain, probably caused by stimulating the larvæ which then burrowed deeper into the tissues.

21-8-14. Three larvæ came out of the patient's nose during the night. His voice was more husky and swallowing was impossible. The headache was still severe and sleeplessness marked. The inflammation on the roof of the mouth had greatly increased and there was a distinct swelling at the junctions of the hard and soft palates.

The nose was washed out with a mixture of chloroform and milk and inhalations of eucalyptus and creosote were given three hourly. Temperature 102.2°F. No more larva came away.

22-8-14. A patch of gangrene about  $\frac{1}{2}$  inch in diameter appeared on the roof of the mouth. The maggots could be seen moving about amongst the dead tissue. Fifteen larvæ were extracted. Temperature 101°F.

23-8-14. The perforation of the palate is now about 1 inch across and 26 larvæ were removed from it with forceps. Pain very severe. Temperature 100°F.

24-8-14. Pain less severe and fluid could be swallowed with difficulty. Twenty-two larvæ were extracted. Temperature normal.

25-8-14. The severe attacks of pain were gone and the swelling of the face was less. No more larvæ were extracted.

In about two weeks' time the perforation on the roof of the mouth had healed and the patient was discharged from hospital.

In all 65 larvæ were recovered from this case and the five found to be these larvæ were identified as *Ptychosema dani* Eck. by Mr. F. W. Edwards.

Da Silva (1912) thinks that the marked dysphagia in cases of nasal myiasis is due to the presence of larvæ in the pharynx and œsophagus, but in my opinion it seems more likely that it is caused by the inflammation of the palate.

*Case No. 2.—(Buccal myiasis).*

A sepoy, aged 35 years, at Kohat, North-West Frontier Province, India. The patient came to hospital complaining of severe pain in the upper jaw and stated that 'worms were eating into his gums.'

His breath was extremely foul-smelling and his upper lip was very swollen. There was a severe pyorrhœa alveolaris of both jaws, but especially the upper. The gums had receded from the roots of the teeth and there were numerous pockets of pus.

On examination numerous larvæ could be seen moving about actively beneath the flesh of the gums at the roots of the upper incisor teeth.

Sixteen larvæ were removed and the gum condition treated.

Mr. F. W. Edwards examined the flies hatched from these larvæ and reported that they were 'a *Pycnosoma* much resembling *P. dux* except in the formation of the eye facets. We have a number of specimens of it from Queensland; but have not been able to find a name for it.'

The larvæ from this case were unfortunately lost, so I am unable to give a description of it.

This case seems of special interest, as I can only find references in literature to three other cases of myiasis of the mouth. Munk recorded maggots in the mouth and Brandt (1888) observed maggots in the gums of a sick person.

Timpano (1914) reported a case of myiasis of the gums in Italy in a patient with mercurial gingivitis.

*Case No. 3.—(Cutaneous myiasis in man).*

A woman, aged 25 years, in the Civil Hospital, Parachinar, Kurram Valley, North-West Frontier Province, India.

This case came to hospital suffering from severe burns over the body and arms. The wounds were more marked over the hands where the deep muscles and tendons were involved.

The fingers were contracted and in the sloughing tissue of the palms a large number of maggots were to be seen.

These were removed with forceps. In all 45 larvæ were removed. The flies which hatched out from these were identified by Mr. F. W. Edwards as *Lucilia serricata*, Mg., the common 'sheep-maggot fly' found in Europe.

*Case No. 4—(Cutaneous myiasis in a dog).*

At Bannu, North-West Frontier Province, India, in May, 1917, a number of fly larvæ were removed from a septic wound behind the ear of a dog. The larvæ were of two types, a large and a small.

Dr. Guy Marshall identified the flies which hatched out from the large type as *Sarcophaga ruficornis*, F., and those from the small type as *Chrysomya dux*, Esch.

*Case No. 5—(Cutaneous myiasis of a camel).*

In April 1918, a number of maggots were removed from a wound in the knee of a camel at Rushkar, near Turbat-i-Haidiri, Khorasan Province, Persia.

The flies from these maggots were identified by Major E. E. Austen as *Wohlfahrtia* sp.

*Case No. 6—(Cutaneous myiasis in a camel).*

In June 1918, at Meshed, Khorasan Province, Persia, a number of fly larvæ removed from a septic wound in a camel's nose caused by the nose-peg hatched out in flies identified by Major Austen as *Sarcophaga* sp. The larvæ from this case were lost unfortunately.

## 5. TECHNIQUE.

The living larvæ were removed from the lesions with as little injury as possible. It is better to remove the larvæ, if possible, before any antiseptic has been used in order that their vitality may not be effected. If an antiseptic has been used, it is better to wash the larvæ quickly in normal saline solution after removal.

The larvæ were then placed in a wide-mouthed bottle or jar containing five or six inches of clean dry sand, on the surface of which a piece of meat has been placed. The utmost care must be exercised that other flies do not deposit their eggs or larvæ on this meat. The mouth of the bottle or jar is covered with two or three layers of very fine gauze both to prevent the adult flies from escaping and what is very important to prevent other flies from infecting the meat. In this connection it should be remembered that some of the *Sarcophagidae* which are *Lixiviparous* can deposit their larvæ from a distance of several inches.

If a piece of slough can be taken from the lesion and placed on the meat, it is an advantage.

When placed in the bottle the larvæ hasten to get away from the light; if mature, they immediately burrow into the sand; if immature, they hide under the meat.

The mature larvæ usually burrow down three or four inches into the sand and remain quiescent for a day or two before they turn into pupæ.

After all the larvæ have burrowed into the sand the meat should be removed. When the adults hatch out, they should be allowed to remain at least 24 hours before being killed and mounted, as the characteristic colours take some time to develop.

The larvæ are best preserved by dropping them into boiling water to kill them and make them extend at the same time, from this they are transferred to 70 per cent alcohol or 10 per cent formalin.

Permanent preparations of various parts, such as stigmata, sclerites, etc., can be prepared by cutting away a part of the larvæ containing the desired structure. An old safety-razor blade is very useful for this purpose. (MacGregor.)

The piece is then boiled for a minute in 10 percent caustic potash solution and allowed to soak for 5 or 10 minutes. The required part can then be dissected out, freed from adventitious tissue, washed in water, dehydrated, cleared and mounted in balsam.

In making preparations of the posterior end to show the tubercles better results were obtained by cutting off this part and simply dehydrating, clearing, and mounting in balsam as there was less distortion of the parts, because the tissue, which would be softened by the caustic, remains as a support.

#### 6. DESCRIPTION OF THE SPECIES OF FLY FOUND.

##### (A). *Pycnosoma* Dux, Esch.

i. Larval stage.—The larvæ removed from the above case of nasal myiasis pupated on the day following their removal from the body.

The larva (Plate VII, Fig. 1) is a footless maggot, thin-skinned and glistening white in colour. When examined with the naked eye, the most conspicuous objects are the black hook-like mandibular sclerites at the anterior end and the brown stigmal plates on the obliquely truncated posterior end.

When fully extended, the larvæ varied in length from 14 mm. to 16 mm. and were about 3.5 mm. wide at their broadest part.

There is no properly differentiated head, the body is segmented and the segments are provided with rings of spines which assist the larvæ in locomotion.

EXPLANATION OF PLATE VII.

The figures were drawn with an Abbe camera-lucida and in all the figures the magnification is shown beside the figure.

The larva of *Pycnosoma dur.* Esch.

- Fig. 1. Lateral view of the larva.  
Fig. 2. Ventral view of anterior end of larva.  
M. P. 'Sensory' papille.  
M. S. Hooks of mandibular sclerites.  
S. D. 'Stomal disc.'  
M. Mouth.  
A. S. Anterior spiracles.  
Fig. 3. Lateral view of anterior end of larva.  
O. L. 'Oral lobes.'  
P. C. 'Pseudo-cephalon.'  
N. S. Second cephalic or 'Newport's segment.'  
1. T. First thoracic segment.  
2. T. Second thoracic segment.  
Other letters as in Fig. 2.  
Fig. 4. Lateral view of anterior end of larva in the contracted condition.  
Fig. 5. Section through grooves of 'stomal disc.'  
Fig. 6. Anterior spiracles.  
Fig. 7. Anterior spiracles showing commencement of tracheae (T).  
Fig. 8. Cephalo-pharyngeal sclerites.  
M. S. Mandibular sclerite.  
D. S. Dentate sclerite.  
P. S. Parastomal sclerite.  
H. S. Hypostomal sclerite.  
L.P.S. Lateral pharyngeal sclerite.

# EXPLANATION OF PLATE / II.

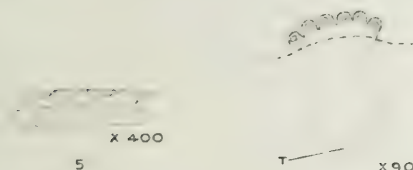
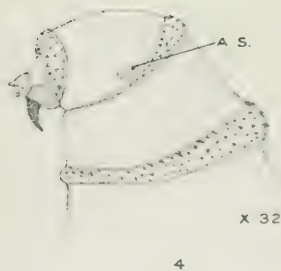
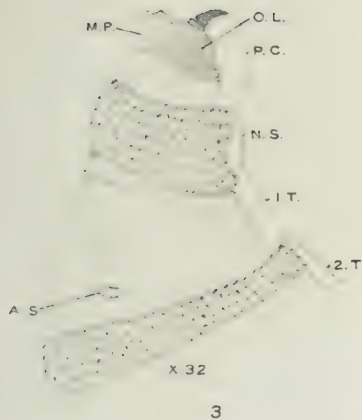
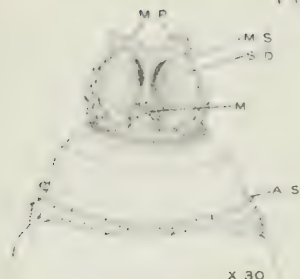
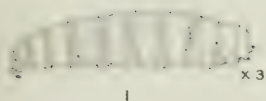
The figures were drawn with an 111/2 camera-lucida and in all the figures the magnification is shown beside the figure.

The larva of *Yponomeuta caryi* Pasch.

- Fig. 1. Lateral view of the larva.
- Fig. 2. Ventral view of anterior end of larva.
  - M. P. Sensory papillae.
  - M. S. Hooks of mandibular sclerites.
  - S. D. Stomal disc.
  - M. Mouth.
  - A. S. Anterior spiracles.
- Fig. 3. Lateral view of anterior end of larva.
  - O. L. 'Oral lobes'.
  - P. C. Pseudo cephalon.
  - N. S. Second cephalic or Zerkow's segment.
  - I. T. First thoracic segment.
  2. T. Second thoracic segment.

Other letters as in Fig. 2.
- Fig. 4. Lateral view of anterior end of larva in the contracted condition.
- Fig. 5. Section through groove of stomal disc.
- Fig. 6. Anterior spiracles.
- Fig. 7. Anterior spiracles showing commencement of trachea. (T).
- Fig. 8. Cephalo-pharyngeal sclerites.
  - M. S. Mandibular sclerite.
  - (D. S. Dentate sclerite.
  - P. S. Poststomal sclerite.
  - H. S. Hypostomal sclerite.
  - L. P. S. Lateral pharyngeal sclerites.

# PLATE VII.





The anterior half of the body is conically cylindrical, while the posterior portion is like a cylinder with an obliquely truncated extremity. The larva is slightly flattened dorso-ventrally.

(a) *The cephalic segments.*

The first segment (Plate VII, Figs. 2, 3 and 4) for which Henneguy's term "pseudo-cephalon" is very suitable, probably represents a much reduced and degenerate cephalic segment, its present form being best suited for the animal's mode of life.' (Hewitt 1910.)

This segment is conical in shape and is bent towards the under surface of the larva, so that the mouth is directed ventrally. It carries none of the spines found on the other segments.

When seen from the ventral aspect, the pseudo-cephalon has a more or less oval appearance. (Plate VII, Fig. 2.) It consists of two rounded lateral portions (the 'oral lobes' of Hewitt) continuous with each other behind but separated in front by the mouth and the grooves in which the mandibular sclerites lie.

The ventral surface of each oral lobe is chiefly occupied by a hemispherical area named by Lowne (1890) the 'stomal disc,' and which is covered by series of fine grooves which converge towards the mouth. If these grooves are followed distally, they will be found to branch dichotomously and to encroach partly on the lateral surfaces of the oral lobes. (Plate VII, Figs. 2 and 3.)

In section these grooves (Plate VII, Fig. 5) are seen to form a series of partly covered channels one edge of which overhangs the other. These channels are  $6\mu$  to  $7\mu$  in diameter and the distance between the centres of adjacent grooves is about  $10\mu$ .

It is along these channels that fluid material runs into the mouth, and Lowne (1890) thinks that they also serve to distribute some secretion over the food. They may be compared to the pseudo-tracheae of the house fly and probably perform a similar function.

Arising from the anterior portions of the oral lobes on their dorsal aspects are two hemispherical projections (Plate VII, Figs. 2 and 3 M.P.) which are separated by a deep groove in the middle line. Each of these carries two mamma-like papillae on its apex, one behind the other. The posterior papilla (Plate VIII, Fig. 10) is surmounted by a cylindrical projection, from a concavity in the end of which a short conical structure projects. The anterior papilla (Plate VIII, Fig. 9) has a small raised ring of chitin on its summit, the upper

of which appears a flattened plate with a few small projections on it. In cleared and mounted specimens, the structures described above are seen to be connected with oval masses in the interior of the papillæ, probably of a ganglionic character.

These papillæ are probably sensory organs of an unknown nature. Very similar structures have been described by Kcilin (1917) in the larva of *Melanochelia riparia* and he considers the posterior structures to be the antennæ and the anterior the maxillary palp. Lowne (1890) believes that the 'oral lobes' with these sensory projections each represent a two-jointed maxilla, but Hewitt (1910) states that their 'homology with the maxillæ is very problematic and at present is not safely tenable.'

The mandibular sclerites or 'great hooks' (Plate VII, Fig. 2 M.S., and Fig. 8 M.S.) when retracted lie in two narrow grooves on the ventro-median surface of the oral lobes. These grooves are separated by a process shaped like an inverted Y with an elongated stem. The limbs of the Y help to form the posterior boundary of the mouth. At the upper end of this process are two small papillæ.

The mouth (Plate VII, Fig. 2 M) is more or less triangular in shape. The base or posterior margin is formed by the labium, which consists of two small lateral lobes and a larger central one. The grooves of the stomal discs unite to form larger channels which enter the mouth near the lateral angles, but there is no apparent connection between the mouth and the grooves in which the 'great hooks' lie.

When retracted the 'pseudo cephalon' is almost entirely withdrawn inside a collar formed by the next segment (Plate VII, Figs. 2 and 4). When the mandibular sclerites are retracted, the oral surface forms a shallow hollow for the reception of foodstuffs with the mouth at its deepest part, but when they are protruded the oral surface becomes flat or even convex.

The functions of the great hooks seem to be two in number; firstly, to assist in locomotion by acting as tractors; and, secondly, to draw food towards the mouth.

The cephalo-pharyngeal sclerites (Plate VII, Fig. 8). These consist of (1) the mandibular sclerites (M.S.); (2) the hypostomal sclerites (H.S.); (3) the dentate sclerites (D.S.); (4) the lateral pharyngeal sclerites (L. P. S.); and (5) the parastomal sclerites (P.S.). These sclerites consist of dark brown or black chitin except for the ends of the posterior

processes of the lateral sclerites which are formed of almost colourless chitin.

(1) The mandibular sclerites or 'great hooks.' The homology of these is uncertain but Lowne (1890) believes them to represent the retractile hooks of the maxillae, while other observers think that they are fused mandibles.

They lie in grooves on the inner margin of the stomal disc and when extended form very conspicuous objects. In total length they measure about 0.45 mm. Each consists of a black chitinous hook with three processes near its posterior end namely, the head, the coronoid and the discoidal processes.

The hook is about 0.28 mm. in length and on its ventral surface near the discoidal process are four small irregularities rather tooth-like in character.

The head is the central of the three processes and articulated by a curved facet with the hypostomal sclerite.

The coronoid or postero-superior process which is not well developed in this larva forms the point of insertion of the mandibular retractor muscle. The discoidal or postero-interior process is closely adherent to the 'stomal disc' on either side of the mouth, and it is because of this attachment that when the maxillary sclerites are drawn towards the mouth, the surface of the 'stomal disc' is also depressed and the cup-like hollow is formed for the reception of food.

(2) The hypostomal sclerite is composed of an irregular vertical plate on either side united by a ventral crossbar of chitin. It articulates in front with the mandibular and posteriorly with the lateral pharyngeal sclerites.

(3) The dentate sclerites are more or less triangular pieces of chitin which articulate with the infero-lateral surfaces of the mandibular sclerites just behind the discoidal process. The mandibular depressor muscle is attached to it.

(4) The lateral pharyngeal sclerites each consist of a body and four processes of which two run directly forward and two directly backwards. The body is squarish and is united with its fellow of the opposite side by a ventral bar of chitin. The antero-superior process is small and hook-like and is separated by a notch from the antero-inferior process which is square and articulates with the hypostomal sclerite. The inferior process by its upper angle in front also affords an attachment for the parastomal sclerite. The postero-superior process is wing-shaped

and is much longer than the postero-inferior process from which it is separated by a deep notch.

The postero-inferior process may have an oval perforation in the dark chitinous portion or may be composed of two wings. It is united with its fellow of the opposite side by a narrow ventral bar.

(5) The parastomal sclerites are narrow curved rods which run upwards and forwards from the antero-inferior process of the lateral pharyngeal sclerites. The retractor muscle of the mandibular sclerite takes origin from it.

The second cephalic segment (called 'Newport's segment' by Lowne) is cylindrical in shape and covered completely by minute spines pointing backwards. It is separated from the first thoracic segment by a faint sulcus. It is within this segment that the 'pseudo-cephalon' can be retracted.

(b) *The thoracic segments.*

These are three in number. The first thoracic segment has a semilunar patch of spines on its antero-ventral margin which extend for a short distance on to the sides also. This segment is only separated by a faint sulcus from 'Newport's segment' and when the larva is contracted these two segments appear to be continuous.

The rest of this segment is smooth and is separated from the spinous anterior border of the second thoracic segment by a faint groove.

The anterior spiracles are situated on the sides of the first segment near its posterior margin. They are two in number and are fan-shaped with a castellated free margin. (Plate VII, Figs. 6 and 7). They arise out of two oval hollows. Their free margin carries 6 papillæ (which are about  $32\mu$  in diameter), on the top of each of which is an oval opening  $26\mu$  across. These openings are surrounded by a ring of chitin from which a fringe of minute spicules project into the lumen to prevent the entrance of extraneous particles into the trachea. In cleared and mounted specimens the openings are seen to run into a common tube shaped like an hour-glass. (Plate VII, Fig. 7.) The anterior spiracles are only developed at the second ecdysis.

The other two thoracic segments have a well-marked collar of spines running completely around their anterior margins, while the remainder of the segments is smooth.

The spines of these segments point backwards and are rather irregularly arranged, but in some cases four or five rows can be made out.

(c) *The abdominal segments.*

These are 9 in number, it is, however, a disputed point whether the ninth or anal segment should be considered a separate segment or only part of the eighth but this will be discussed later.

The widest part of the body is at the third and fourth abdominal segments and from this the body tapers gradually towards the anterior end but less markedly towards the posterior extremity. The last two segments are much smaller than the preceding ones.

Each segment is separated from the adjacent ones by a groove running round the body. The anterior portion of each segment is surrounded by a ring of spines which are absent from the posterior part.

The first abdominal segment resembles the thoracic ones. The next seven segments differ from the first in the more marked development of the spinous ring, especially on the ventral surface, and in the arrangement of the spines. The ventral portion of the ring is wider and is divided into two parts by a transverse groove.

On the lateral surfaces of the rings there is a small oval portion cut off from the main ring by a curved groove.

The dorsal portion is undivided. The abdominal and thoracic segments have also a number of low tubercles which are shown diagrammatically along with the spinous areas in text figure 1.

TEXT FIG. 1.

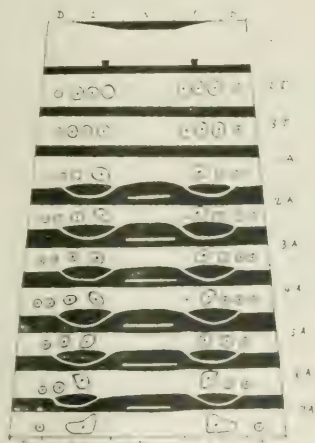


Diagram of the spinous areas and tubercles on the body of the larva of *P. duvali*. The dark areas represent the spines and the circles the tubercles. D. is the dorsal surface, L. is the lateral surface, V. is the ventral surface. The other letters and figures denote the segments.\*

\* In the specimens from which these diagrams were made the head and posterior extremities were removed and the body wall was flattened. The tubercles were then treated with caustic potash to remove the chitinous part, removed, after which the thin chitinous wall was spread out and flattened.

The posterior end of the body is formed by the eighth and ninth segments.

The eighth abdominal segment like the 'pseudo cephalon' can be retracted inside a collar formed by the seventh segment, from which it is separated by a distinct groove. The anterior ring of hooklets is not so well marked as in the other abdominal segments.

This segment has on its postero-superior surface a saucer-like hollow which looks upwards and backwards at an angle of  $150^{\circ}$  to  $170^{\circ}$  to the long axis of the body. Text figure 2 is a diagrammatic representation of a median sagittal section through this hollow. It is in the posterior part of this hollow that the posterior spiracles lie.

TEXT FIG. 2.

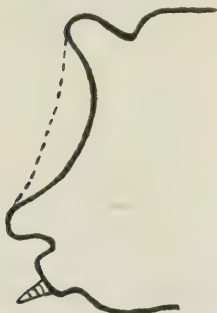


Diagram of median section through the posterior end of the larva of *P. duar*.

The hollow (Plate VIII, Fig. 12) is surrounded by a thick lip carrying a series of pointed tubercles, and this lip is cut off from the rest of the segment by a circular sulcus. (Plate VIII, Fig. 13.)

The hollow is divided into a ventral and a dorsal portion by a transverse groove. In the ventral part, which is placed at an angle of about  $120^{\circ}$  to the dorsal, are two circular depressions between which a flat area runs back to the transverse groove.

These depressions probably represent the attachment of muscles.

The stigmal plates lie in the dorsal part of the hollow. In the full-grown larva the plates are more or less fan-shaped chitinous areas slightly raised above the surrounding tissue. (Plate VIII, Figs. 12 and 15.)

Their external and dorsal surfaces form a rounded margin, while the internal and the ventral edges are nearly straight.

The distance between the outer margins of the plates is 0.79 mm. to 0.82 mm., and between the inner margins from 0.050 mm. to 0.070 mm.

The margin of the stigmal plate is composed of a well-marked ring of dark brown chitin which becomes very faint at the junctions of the internal and ventral margins—the 'hilum.'

At this point a small oval area can be made out, which has been called the 'button,' this is poorly developed in this larva (cf. *Lucilia serricata*); this area probably represents a muscular attachment. The distance between the centres of the buttons is about 0.18 mm.

# EXPLANATION OF PLATE VIII.

The larva and pupa of *Ptychosomus dani*, Esch.

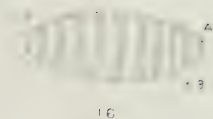
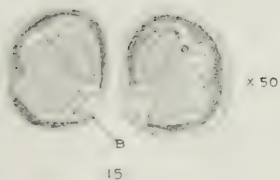
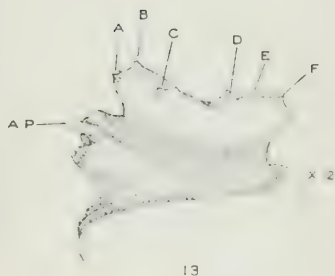
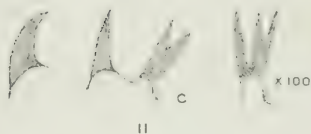
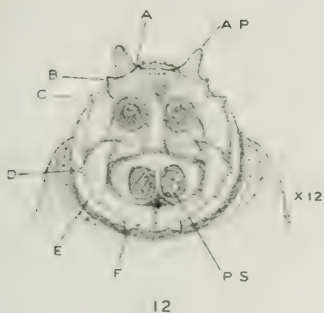
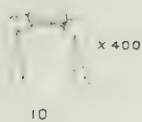
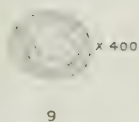
- Fig. 9. The sensory (?) organ on the anterior 'sensory' papilla on the 'pseudo-cephalon.'
- Fig. 10. The sensory (?) organ on the posterior papilla of the 'pseudo-cephalon.'
- Fig. 11. Spines from the larva.  
 (a) Spine from abdominal rings.  
 (b) Spines from 'posterior hollow.'  
 (c) Spines from margins of 'posterior hollow.'
- Fig. 12. Posterior view of 'posterior hollow' of 8th abdominal segment of larva.  
 A. P. Anal papillae.  
 A. B. and C. Tubercles on ventral edge of 'posterior hollow.'  
 D. E. and F. Tubercles on dorsal edge.  
 P. S. Posterior stigmal plate.
- Fig. 13. Lateral view of 8th abdominal segment.  
 Letters as in Fig. 12.
- Fig. 14. Ventral view of 8th and 9th abdominal segments.  
 P. R. Post-anal ridge.  
 Other letters as in Fig. 12.
- Fig. 15. Posterior stigmal plates of larva.  
 B. The 'button.'
- Fig. 16. Ventral view of pupa.  
 A. Anterior end.  
 P. Posterior end.

# EXPLANATION OF PLATE VIII.

The letters and figures of *Yucca elaeagnifolia* Fisch.

- Fig. 9. The sensor (1) organ on the anterior papilla on the 'pseudo-cephalon'.
- Fig. 10. The sensor (2) organ on the posterior papilla of the 'pseudo-cephalon'.
- Fig. 11. Spines from the larva  
(a) Spine from abdominal ring.  
(b) Spines from posterior hollow.  
(c) Spines from margins of posterior hollow.
- Fig. 12. Posterior view of posterior hollow of sub-abdominal segment of larva  
A. P. *Posterior papilla*  
A. B. and C. *Tubercles on central edge of posterior hollow.*  
D. E. and F. *Tubercles on dorsal edge.*  
P. S. *Posterior subsegment.*
- Fig. 13. Lateral view of sub-abdominal segment  
Posterior as in Fig. 12.
- Fig. 14. Ventral view of sub-abdominal segment.  
P. R. *Posterior ridge.*  
Other letters as in Fig. 12.
- Fig. 15. Posterior abdominal plates of larva  
B. The *intima*  
C. *Ventral view of larva.*  
A. *Anterior end.*  
P. *Posterior end.*

# PLATE VIII.





In each plate there are three separate slits opening into the tracheae. These are sausage shaped and run upwards and dorsally from the bottom and in the following descriptions they will be called the internal, the central and the external slits for ease of reference. The central slit is slightly the longest and varies in length from 0.25 mm. to 0.30 mm. and in breadth from 0.08 mm. to 0.10 mm. This slit runs at an angle of about 45° to the median sagittal plane (varying from 40° to 50° in ten specimens examined).

These stigmal orifices have a well-marked thickened margin of dark brown chitin, and from each side of the opening run out a series of projections arranged in two layers.

The outer layer consists of a series of spines projecting into the opening and numbering from 25 to 30 on either side of the central slit and from 20 to 25 in the lateral slits. From the under surface of these outer bars run downwards to anastomose with the inner layer of bars; the former bars also send branches to the bars on either side of them, so making a series of vertical and horizontal loops.

The inner layer consists of a series of stout chitinous bars which run across the slits from side to side. The bars may be branched or unbranched and the branches usually anastomose with the adjacent bars.

The branching is not however regular, so no definite pattern is formed.

The main bars number from 16 to 18 in the central slits and from 13 to 16 in the lateral ones. There are no small spicules projecting from the bars as seen in *Sarcophaga ruficornis*.

The posterior hollow is surrounded by a thickened lip which carries a series of twelve tubercles of varying size. (Plate VIII. Figs. 12, 13 and 14.) These tubercles may be divided into those on the ventral portion and those on the dorsal. On the ventral part are six tubercles arranged in pairs—(A) a central pair of small blunt papillae lying almost vertically beneath the centres of the stigmal plates, (B) a large lateral pair of a mamillary type with a small nipple-like projection on the summit and with a base surrounded by concentric rings, and (C) a medium-sized pair similar to the last but situated more dorsally and separated from them by an oblique groove. On the dorsal portion of the edge are three more pairs of tubercles—(D) a large pair similar to (B) and situated opposite the ventral edge of the stigmal plates, (E) a small papilla-like pair on the dorsal side of the bases of the last pair, and (F) a dorso-central pair very like (C) in size and shape.

The ventral portion and the margins of the hollow are covered with a series of small spines varying greatly in size, those on the hollow being the smaller (Plate VIII, Fig. 11 b.) and those on the outer edges of the lip the larger and may be bifid or trid (Plate VIII, Fig. 11 c.). The spines are arranged in semicircular groups of 5 to 10, those on the hollow being more or less transverse, while those on the margin are more or less parallel to it. At the base of the tubercles they tend to form rings around them.

*The ninth or anal segment.* This segment is attached to the ventral surface of the eighth segment and, when the posterior end of the larva is fully expanded, this segment and the ventral lip of the hollow form a marked projection. (Plate VIII, Fig. 13.)

The base of this segment is surrounded by a ring of spines which extends on to the ridge between the anal appendages. (Plate VIII, Fig. 14.)

The most conspicuous objects in this segment are the two anal appendages each of which has a conical base surmounted by a horn-like projection ending in a small pointed papilla. The base is separated by a distinct sulcus from the horn-like projection which is surrounded by a series of faint rings. (Plate VIII, Fig. 14.) The bases are joined by a transverse ridge behind the anus. Between this ridge and the anus lies a shallow hollow.

The anus, which is directed ventrally, is a vertical slit with a thick lip on either side.

Whether the anal segment of Muscoid larvæ is a separate segment or only part of the eighth segment of the abdomen is disputed. Hewitt (1910) thinks that it is not, but Lowne (1890) supports Brauer (1882) in believing it to be a separate segment, because 'the complex nature of the last segment is not, I think, doubtful, but is manifest by the existence of a pair of ventral appendages close to the anus each of which consists of three joints and a small ventral plate.' I am inclined to agree with Lowne for the following reasons, because (1) if we examine the other abdominal segments, we find a ring of hooklets at their anterior margins similar to those found on the anal segment in its anterior part; (2) there is a striking resemblance between the posterior end of this larva and that of an anopheline larva in the position of the posterior stigmata and of the anus, and the anal segment of this larva may be taken to represent the 9th abdominal segment of anopheline larvæ and the anal appendages, the ventral papillæ.

## II. THE PUPAL STAGE.

In the cases under consideration the pupal stage lasted 8 to 9 days.

The pupa was of a rich dark brown colour and measured 10 mm. to 12 mm. in length by about 4 mm. in breadth.

When the larva begins to pupate it contracts as a whole, but the contraction is most marked in the case of the anterior segments which are entirely withdrawn until the anterior spiracles come to lie at the anterior end of the body.

Two new pupal spiracles are formed as small spine-like projections dorso-laterally at the junction of the 1st and 2nd abdominal segments.

On the abdominal and thoracic segments the rings of spines can be made out and the contraction of the body is shown by a series of circular ridges running around the body between the rings of spines.

At the posterior end of the body the characteristic stigmal plates, the tubercles around the hollow and the anal appendages can be made out.

The imago escapes from the anterior portion of the pupal case by a circular slit near what corresponds to the anterior margin of the first abdominal segment of the larva. This cap-like portion is usually split into two halves by two lateral cracks running forwards horizontally and meeting at the anterior end.

In the interior of the empty pupa case can be seen the discarded tracheal tubes.

## III. ADULT STAGE.

These are metallic greenish or bluish, thickest flies with characteristic orange red cheeks.

The generic characters of the genus *Pycnosoma*, Brauer and Bergenstamm (1893), are 'a Calliphorine with metallic bluish or greenish body. The third longitudinal vein of the wing is bare or sometimes hairy. The thorax is without longitudinal dark bands. In the male the facets of the upper  $\frac{2}{3}$  or  $\frac{3}{4}$  of the eye are slightly larger.' (Brumpt 1913.)

Patton and Cragg (1913) also mention the following generic characters:—'Closely allied to *Lucilia*. Cheeks usually buff or orange red. Posterior dorso-central and acrostichal bristles well developed. Sternopleural bristles only two in number arranged 1:1.'

The identification of the different species is a difficult matter and for accuracy it is advisable to send specimens of the adult male and female flies to Major E. E. Austen, D.S.O., British Museum, Natural History, South Kensington, London.

(B.) *Lucilia serricata*, Mg.

(c). Larval stage.

These larvae were obtained from case No. 3, and pupated 6 days after removal from the body.

In length they varied from 11 mm. to 16 mm. and were about 3 mm. in breadth.

The general morphology of the larva is very similar to that of *P. dux* Esch. as described above.

The anterior end differed from that of *P. dux* in the following points (Fig. 18):—

(a) The small papillae at the base of the Y-shaped process in *P. dux* are situated on the upper part of the 'stomal discs' in this species.

(b) The ring of spines at the anterior margin of the first thoracic segment are better developed here.

(c) The anterior spiracles (Plate IX, Fig. 19) have 8 openings in contradistinction to the 6 seen in *P. dux*.

The other thoracic and abdominal segments differ from those of *P. dux* in the arrangement of the spines and tubercles (Text Fig. No. 3).

TEXT FIG. 3

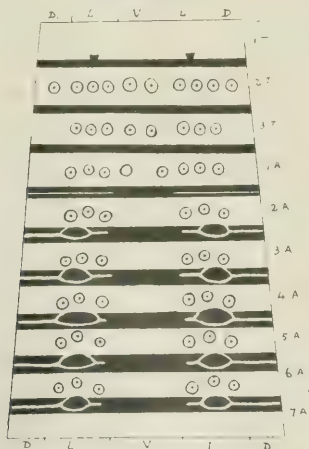


Diagram of the spinous areas and tubercles on the body of the larva of *L. serricata*. The dark parts represent the spinous areas and the circles the tubercles. D, is the dorsal surface. L, is the lateral surface. V, is the ventral surface. The other letters and figures denote the segments.

EXPLANATION OF PLATE IX.

The larva and pupa of *Lucilia serricuta* Mg

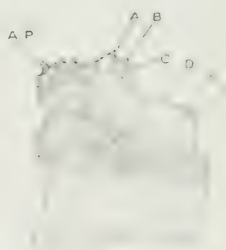
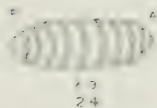
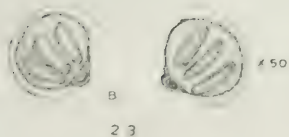
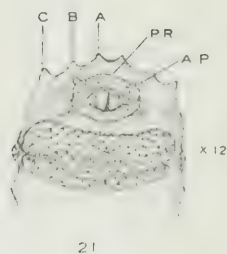
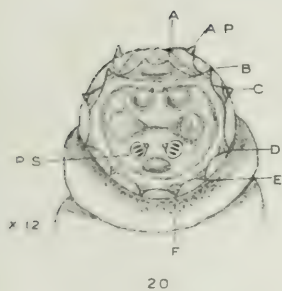
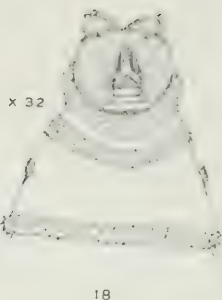
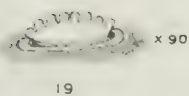
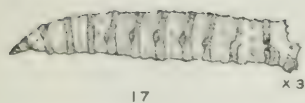
- Fig. 17. Lateral view of the larva.
- Fig. 18. Ventral view of 'pseudo-cephalon' and 1st thoracic segment of the larva.
- Fig. 19. Anterior spiracles
- Fig. 20. Dorsal view of 'posterior hollow' of 8th abdominal segment of the larva.  
A. P. Anal papilla.  
P. S. Posterior stigmal plate.  
A., B. and C. Tubercles on ventral edge of 'posterior hollow'  
D., E. and F. Tubercles on dorsal edge.
- Fig. 21. Ventral view of 8th and 9th abdominal segments of the larva.  
P. R. Post-anal ridge.  
Other letters as in Fig. 20.
- Fig. 22. Lateral view of posterior end of larva.  
Letters as in Figs. 21 and 22.
- Fig. 23. Posterior stigmal plates of larva.  
B. The 'button.'
- Fig. 24. Ventral view of pupa.  
A. Anterior end.  
P. Posterior end.

# EXPLANATION OF PLATE LX.

The larva and pupa of *Lucilia sericata* Mg.

- Fig. 17. Lateral view of the larva.  
 Fig. 18. Ventral view of 'pseudo-cephalon' and 1st thoracic segment of the larva.  
 Fig. 19. Anterior spiracles.  
 Fig. 20. Dorsal view of 'posterior hollow' of 8th abdominal segment of the larva.  
 A. P. Anal papilla.  
 P. S. Posterior stigmatal plate.  
 A. B. and C. Tubercles on ventral edge of 'posterior hollow'.  
 D. E. and F. Tubercles on dorsal edge.  
 Fig. 21. Ventral view of 8th and 9th abdominal segments of the larva.  
 P. R. Post-anal ridge.  
 Other letters as in Fig. 20.  
 Fig. 22. Lateral view of posterior end of larva.  
 Letters as in Figs. 21 and 20.  
 Fig. 23. Posterior stigmatal plate of larva.  
 B. The 'button'.  
 Fig. 24. Ventral view of pupa.  
 A. Anterior end.  
 P. Posterior end.

# PLATE IX.





The tubercles are better developed and more conspicuous in the larva of *L. serricata*.

It is at the posterior end however that the most marked differences are found in the two species.

The dorsal part of the 8th abdominal segment has a similar depression to that found in *P. dur*, but instead of being saucer like it is more like the seat and back of a chair. (Plate IX, Fig. 22 and Text Fig. 4.) The

TEXT FIG. 4.



Diagram of median section  
through the posterior end of  
the larva of *L. serricata*.

ventral part is set at an angle of  $135^{\circ}$  to the dorsal part which is transverse to the long axis of the body. The hollow as a whole looks backwards and upwards. (Plate IX, Fig. 17.)

The edge of the hollow is not so thick and rounded as in *P. dur* and is not surrounded by the same wide groove.

The edges of the hollow are covered with spines each on a small conical base. In the ventral part of the hollow, however, the conical bases are present but they have no spinous pointed extremities.

There are two small spine-like papillæ in the ventral part of the posterior hollow which have not been noted in the larva of *P. dur*.

The tubercles around the lip of the hollow are twelve in number (Plate IX, Figs. 20, 21, and 22) and vary markedly from those in the larva of *P. dur*.

On the ventral part of the edge are six tubercles—(A) a small central pair, (B) a medium-sized lateral pair rather on the inner edge of the lip, these pairs are joined by a faint ridge across the ventral part of the posterior hollow, and (C) a large pair almost in line with or slightly posterior to the latter pair but placed on the outer edge of the lip.

The three pairs of tubercles (D, E and F) on the dorsal part of the lip are all of medium size and are distributed equi-distantly along this part of the rim.

The posterior spiracles are markedly different in the two species. In both they are rather fan-shaped, but in *L. serricata* they are smaller and placed further apart.

The distance between the outer margins is about 0.80 mm. and between the inner 0.26 mm. to 0.30 mm.

The margins of the stigmal plates have a more delicate but well-marked dark brown chitinous ring which divides at the ventro-internal angle to enclose an oval mass of light brown chitin—the 'button'—which has a lighter centre. (Plate IX. Fig. 23.) The button is much better marked in this species than in *P. dux*. The distance between the centres of the 'buttons' varies from 0.27 mm. to 0.33 mm.

The slits in the plates are three in number on each side and run dorsally and outwards from the 'button.' They are sausage-shaped, slightly wider at the centre than at the ends and surrounded by a mere delicate ring than in *P. dux*. The central one is slightly the longest, measuring from 0.20 mm. to 0.25 mm. in length and makes an angle of about 45° with the median sagittal plane.

The chitinous projections in the slits are arranged as in *P. dux*, but the outer layer of spines numbers from 22 to 26 on each side of the slit and the inner from 16 to 20.

If this is compared with the description and figures of *L. caesar* given by MacGregor (1914) it will be seen that in *L. caesar* the distance between the buttons is wider (0.40 mm. to 0.48 mm.), the central slit makes an angle of 40° with the middle line and the number of bars in the slits is from 11 to 14.

The anal papillæ are smaller than in *P. dux* and there is not the marked hollow between the post-anal ridge and the anus. The post-anal ridge has no cleft.

#### (ii). Pupal stage.

The pupal life lasted 9 days.—The pupa was 8 mm. to 9 mm. in length and about 3.35 mm. broad.

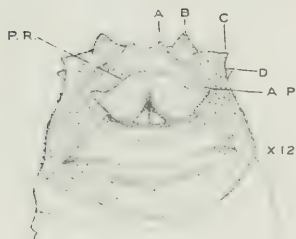
In appearance it is very like the pupa of *P. dux*, but the anterior spiracles,\* the arrangement of the spines on the body, the shape, etc., of the posterior stigmata and the size and shape of the tubercles around the posterior hollow distinguish it from that species. (Plate IX. Fig. 24.)



# PLATE X.

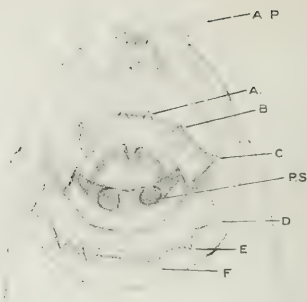
x 90

25

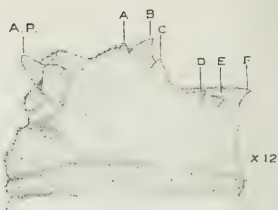


27

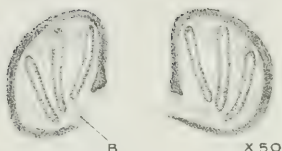
x 12



26



28

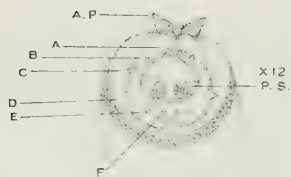


29

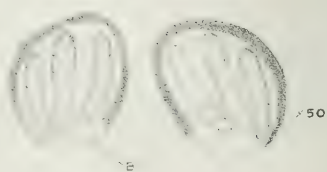
x 90



30



31



32

# EXPLANATION OF PLATE X.

The series of *Staphylinus* *eximius* F. and *Staphylinus* sp.  
 taken at *Staphylinus* F.

- Fig. 25. Anterior spiracles.
- Fig. 26. Posterior view of posterior hollow.
- Fig. 27. *Staphylinus*.
- Fig. 28. *Staphylinus*.
- Fig. 29. *Staphylinus*.
- Fig. 30. *Staphylinus*.
- Fig. 31. *Staphylinus*.
- Fig. 32. *Staphylinus*.
- Fig. 33. *Staphylinus*.
- Fig. 34. *Staphylinus*.
- Fig. 35. *Staphylinus*.
- Fig. 36. *Staphylinus*.
- Fig. 37. *Staphylinus*.
- Fig. 38. *Staphylinus*.
- Fig. 39. *Staphylinus*.
- Fig. 40. *Staphylinus*.
- Fig. 41. *Staphylinus*.
- Fig. 42. *Staphylinus*.
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- Fig. 44. *Staphylinus*.
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- Fig. 48. *Staphylinus*.
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- Fig. 61. *Staphylinus*.
- Fig. 62. *Staphylinus*.
- Fig. 63. *Staphylinus*.
- Fig. 64. *Staphylinus*.
- Fig. 65. *Staphylinus*.
- Fig. 66. *Staphylinus*.
- Fig. 67. *Staphylinus*.
- Fig. 68. *Staphylinus*.
- Fig. 69. *Staphylinus*.
- Fig. 70. *Staphylinus*.
- Fig. 71. *Staphylinus*.
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- Fig. 92. *Staphylinus*.
- Fig. 93. *Staphylinus*.
- Fig. 94. *Staphylinus*.
- Fig. 95. *Staphylinus*.
- Fig. 96. *Staphylinus*.
- Fig. 97. *Staphylinus*.
- Fig. 98. *Staphylinus*.
- Fig. 99. *Staphylinus*.
- Fig. 100. *Staphylinus*.

EXPLANATION OF PLATE X.

The larvæ of *Sarcophaga ruficornis*, F. and *Wohlfahrtia*, sp.  
Larva of *S. ruficornis*, F.

- Fig. 25. Anterior spiracles.  
Fig. 26. Posterior view of 'posterior hollow.'  
A. P. Anal papilla.  
P. R. Post-anal ridge.  
A., B. and C. Tubercles on ventral lip of 'posterior hollow.'  
D., E. and F. Tubercles on dorsal lip.  
P. S. Posterior stigmal plates.  
Fig. 27. Ventral view of 8th and 9th abdominal segments.  
Letters as in Fig. 26.  
Fig. 28. Lateral view of posterior end of body.  
Letters as in Figs. 26 and 27.  
Fig. 29. Posterior stigmal plates.  
B. The 'button.'  
Larva of *Wohlfahrtia*, sp.  
Fig. 30. Anterior spiracles.  
Fig. 31. Posterior view of 'posterior hollow.'  
Letters as in Fig. 26.  
Fig. 32. Posterior stigmal plates.  
B. The 'button.'

## (iii). Adult stage.

This is a metallic greenish blue fly very like *Pycnosoma* but without the red cheeks.

Brumpt (1913) gives the following as the generic characters of the genus *Lucilia* Robineau-Desvoidy 1830:— 'A Calliphorine with smooth eyes. The first post-marginal cell opens in front of the end of the wing.'

The fourth longitudinal vein is bent at an obtuse angle and rounded. The third longitudinal vein provided with spines in its proximal part in front of the small transverse vein. Thorax and abdomen metallic green or blue, very brilliant with silky reflections.'

Patton and Cragg (1913) give as generic characters: 'Medium-sized flies either green or bluish green. Posterior dorso-central and acrostichal bristles constant and well developed. Sternopleural bristles arranged 2:1. Prothoracic stigmata black. Third longitudinal vein spinulose either at its base or throughout its length.'

Like all the Calliphorinæ this fly is oviparous.

Specimens of the adults should be sent to the British Museum for identification.

(C). *Sarcophaga ruficornis*, F.

## (c). Larval stage.

These larvæ were removed from case No. 4 and pupated 5 days after removal from the wound.

The length of the larva was 20 mm. and the breadth 4 mm.

The cephalic segments were very like those of the last two species.

*The thoracic segments.*—The anterior spiracles have 11 papillæ. (Plate X, Fig. 25.) The anterior portion of each segment has the usual ring of spines. The posterior portion was bare in the first two segments but in the third there was a poor development of spines on the lateral and dorsal surfaces of this portion. (Text Fig. 5.)

*The abdominal segments.*—These segments had the usual anterior ring of spines but on the ventral part it was divided into two by a faint transverse sulcus.

A spinous development similar to that seen on the posterior portions of the third thoracic segment but to a more rounded degree is present here, especially on the more posterior segments of the abdomen which are practically entirely covered with spines on their dorsal and lateral surfaces.

The ventral surface is practically devoid of spines on its posterior parts of the segments except for a very narrow posterior band on all the segments except the first. (Text Fig. 5.) The place of spines is taken by a series of six tubercles running transversely. The posterior segments of the abdomen have also a series of smaller tubercles on their lateral and dorsal surfaces.

XT FIG. 5

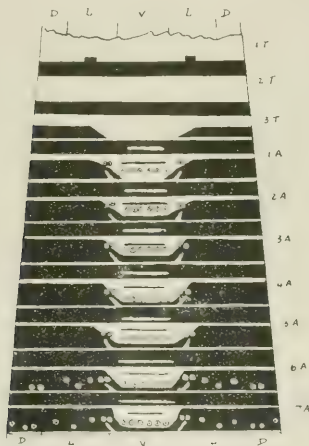


Diagram of the spinous areas and tubercles of the body of the larva of *S. rufo-cornis*. The dark parts represent the spinous areas and the circles the tubercles. D, is the dorsal surface. L, is the lateral surface. V, is the ventral surface. The other letters and figures denote the segments.

*The posterior extremity.*—The posterior hollow is very deep and cup-like. (Text Fig. 6 and Plate X, Fig. 26.)

The ventral part of the edge makes an angle of about  $130^\circ$  with the dorsal part. The hollow looks almost directly backwards. The ventral portion of the hollow runs sharply downwards to the bottom of the cup but bulges forward so that it is difficult to see the stigmal plates. The two depressions in this part of the hollow are rather triangular in shape.

The dorsal part runs directly downwards to the dorsal margins of the stigmal plates (Text Fig. 6), which lie in an oblong depression

TEXT FIG. 6.



Diagram of median section through  
the posterior end of the larva  
of *S. unicornis*.

at the bottom of the cup. Between the plates there is a marked hollow and the transverse groove is well-marked. (Plate X, Fig. 26.)

The anterior ring of spines is well-developed in the 8th abdominal segments and its sides are also thickly clothed with spines. (Plate X, Fig. 28.) The rim of the posterior hollow is covered with spines with a conical base and a fine almost hair-like pointed extremity, but in the central part of the hollow the conical bases are present but have no pointed ends.

The lip of the posterior hollow is divided into a ventral and a dorsal part by a deep groove (Plate X, Fig. 26), and the tubercles on the edge are 12 in number. (Plate X, Figs. 26, 27 and 28.) Those on the ventral part are (A), a small median pair, (B) and (C) large pairs of tubercles dorsal to the last. On the dorsal edge are three pairs also—(D) a large pair just dorsal to the transverse groove on the lip, (E) a medium-sized pair just dorsal to these and (F) a large dorso-central pair.

The stigmal plates have well-marked chitinous rings which are deficient on the ventral portions of their inner margins (the 'hilum') (Plate X, Fig. 29.) The inner side ends in a knob-like thickening while the ventral tails off to a point.

On a more careful examination of mounted specimens it can be made out corresponding to the 'hilum' (Plate X, Fig. 29.)

to find a button in *S. sarraecenia*). The distance between the inner margins of the plates was about 0.17 mm. and between the outer edges about 0.95 mm.

The ' buttons ' were about 0.36 mm. apart from centre to centre.

There are three slits in each plate but these differ from those of the last species in that the internal slit runs *centrally*, and outwards, and the central one is practically parallel to the median sagittal plane. (Plate X, Fig. 29.)

The central slit is the longest and measures about 0.36 mm. The outer row of spines in the slit are like those already described but number from 34 to 37 on each side. The inner layer of transverse bars numbers about 24 and tend to branch and anastomose as described in the other two species, but on careful examination, especially from below in mounted specimens, it will be seen that they have a number of very fine spicules running out from their sides terminating in a point.

The anal segment is very like those described in the other two species but differs in the marked groove which is present on the post-anal ridge. (Plate X, Figs. 26 and 27.)

(ii). The pupal stage.

The pupa measured about 13 mm. in length and about 4 mm. in breadth. It differed from the other species in the number of papillæ on the anterior spiracles, the arrangement of the spines, etc., etc. This stage lasted 10 to 11 day.

(iii). Adult stage

This fly is about ' 12 mm. long, a light grey colour with the usual blackish markings not so dark as is often the case in this genus, while the antennæ and palpi are ochraceous-rufous and the anal segments of the abdomen ferruginous.' (Austen 1910.)

Brumpt (1913) gives the following as the generic characters of the genus *Sarcophaga* Meigen 1826 :—' The aristæ of the antennæ plumose except in the distal  $\frac{1}{4}$ th which is bare. The abdominal segments grey with shot reflections and a chequered pattern. The first posterior cell is open or shut in this case with a short stem. The posterior transverse vein is not placed more obliquely than the terminal transverse vein.'

These flies are larviparous.

In sending specimens for identification a male should always be sent, as it is practically impossible to distinguish the different species in the females.

(D). *Wohlfahrtia* sp.

The larvæ of this species were unfortunately allowed to become dried up before they were examined so the description could not be so thoroughly made out as in the other species.

(i). *1 a v a s t g e.*

The larvæ of this species was obtained from case No. 5. They pupated about 7 days after removal from the body.

In length the larva measured about 11 mm. and in breadth about 3 mm.

On account of the shrunken condition of the larvæ it was impossible to decide whether there was any marked difference in the cephalic region as compared with the other larvæ, but it appeared as if the bases of the sensory tubercles on the head were joined by a ridge which tended to project over the upper part of the 'stomal disc.'

The anterior spiracles had only 5 papillæ. (Plate X, Fig. 30.)

The larva was less spinous than the others described and the spines were more widely distributed in a peculiar pattern. (Text Fig. 7.)

TEXT FIG. 7.

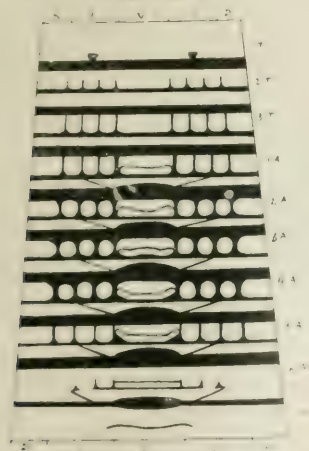


Diagram of the spinous areas on the body of the larva of *Wohlfahrtia*, sp. The dark areas represent the spinous areas. D, is the dorsal area. L, is the lateral area. V, is the ventral area. The other figures and letters denote the segments.

The eighth abdominal segment has none of the marked spines seen in *S. ruficornis*. The posterior hollow has the same cup-like character seen in the last species and looks backwards and very slightly upwards. (Text Fig. 8.)

TEXT FIG. 8.

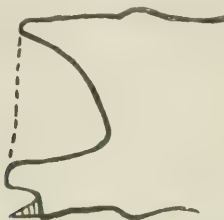


Diagram of median section through  
the posterior end of the larva  
of *Wohlfahrtia* sp.

There are three pairs of tubercles on the ventral part of the edge—(A) a small ventral pair, (B) a medium-sized pair lateral to these and (C) a large pair on the outer edge of the lip near the junction of the dorsal and ventral parts of the edge. (Plate X, Fig. 31.)

There are three pairs on the dorsal edge—(D) a large pair laterally, (F) a small dorso-central pair and (E) a medium-sized pair between (D) and (F). The tubercles were very hard to make out on account of the shrunken condition of the larvæ.

There were the same small spines on the rim of the hollow as described in the other species, but they were finer and more hair-like and did not extend on to the ventral part of the hollow.

The stigmal plates had a heavy chitinous ring, which was deficient on its ventral aspect, at which point on careful examination a very faint circular area corresponding to the 'button' could be made out. (Plate X, Fig. 32.)

The inner part of the ring ended in a bifid thickening, while the outer terminated in a blunt point.

The distance between the outer margins of the plates was about 1.02 mm. and between the inner about 0.107 mm.

The centres of the buttons was about 0.58 mm. apart.

The slits number three in each plate of which the central is the longest being about 0.42 mm. in length.

The internal slits run *centrally* and outwards and the central one in the same direction making an angle of about  $168^\circ$  with the middle line. (Plate X, Fig. 32.)

The spines on the edges of the slits numbered about 31 on each side while the inner layer of bars was about 15 but without any of the fine spicules seen in *S. ruficornis*.

The anal segment differed in the very marked notch in the post-anal ridge. (Plate X, Fig. 31.)

#### (ii). Pupal stage.

The pupa only hatched out after 15 days of pupal life. They measured 8.9 mm. in length and about 3.25 mm. in breadth.

The pupa showed the same differences as those noted in the other species.

#### (iii). Adult stage.

The genus *Wohlfahrtia* Brauer has the following characters:—‘A Sarcophagina in which the arista of the antenna is hairy. The third segment of the antenna about twice as long as the second. The front large in both sexes. The abdomen whitish or grey, with a more or less distinct pattern but without shot reflections.’ (Brumpt 1913.)

Portchinsky (1916) reports *W. balaglossi* Portsch, and *W. tertriperactata* [?]. Duf. from Central Asia, so the species described above may have been one of these.

#### (7) COMPARISON OF THE LARVAL AND PUPAL CHARACTERS.

MacGregor (1914) writing on the distinctive characters of the posterior stigmata of dipterous larvæ states that ‘one genus is distinguished from the other by variations in (1) the orientation of the stigmata with reference to the angle made to the longitudinal axis of the body; (2) in the distance between the stigmata themselves; and (3) in variations of the gross structures, and shape of these organs: such as a thickening or otherwise of the chitinous ring that borders them, etc.’

‘The specific differences are to be found in the quite often remarkable variations that occur in the finer structure, *i.e.*, in the transverse bars of the slits, both as to arrangement and number, as well as the variation in position of the “button.”’

In the present investigation all these points were found to be of great importance but it is considered that attention should also be paid to the number of papillæ on the anterior spiracles; the distribution of

Character.	<i>P. dux</i> , Esch.	<i>L. serricata</i> , Mg.	<i>S. roficornis</i> , F.	<i>Wohlfahrtia</i> , sp.
<b>A. Larva.</b>				
(1) Length .. ..	14 mm. to 16 mm. ..	14 mm.—16 mm.	20 mm. ..	11 mm.
(2) Breadth .. ..	3.5 mm. ..	3 mm. ..	4 mm. ..	3 mm.
(3) Anterior spiracles ..	6 papillae ..	8 papillae ..	11 papillae ..	5 papillae.
(4) Distribution of spines on the body.	See Text Fig. 1 ..	See Text Fig. 3	See Text Fig. 5 ..	See Text Fig. 7.
(5) Posterior hollow—				
(a) Shape ..	Saucer-like ..	Chair-like ..	Cup-shaped ..	Cup-shaped.
(b) Direction ..	Looks backward and slightly upwards.	Looks equally upwards and backwards.	Looks backwards	Looks backwards and very slightly upwards.
(c) Tubercles on the edge.	A. small ..	A. small ..	A. small ..	A. small.
	B. large ..	B. medium ..	B. large ..	B. medium.
	C. medium ..	C. large ..	C. large ..	C. large
	D. large ..	D. medium ..	D. large ..	D. large.
	E. small ..	E. medium ..	E. medium ..	E. medium.
	F. medium ..	F. medium ..	F. large ..	F. small.
(6) Posterior stigmal plates—				
(a) Shape ..	Fan-shaped ..	Fan-shaped ..	Oval ..	Oval.
(b) Direction of 'hilum.'	Inwards and slightly ventrally.	.....	Inwards ..	Ventrally.
(c) Chitin of ring ..	Thick and well-marked.	Delicate but well-marked.	Thick and well-marked ..	Thick and well-marked.
(d) Button ..	Feebly developed ..	Markedly developed.	Practically absent	Practically absent.
(e) Distance between inner edges of plates.	0.05 mm.—0.07 mm.	0.26 mm.—0.3 mm.	0.17 mm. ..	0.107 mm.
(f) Distance between outer edges of plates.	0.79 mm.—0.82 mm.	0.8 mm. ..	0.95 mm. ..	1.02 mm.
(g) Distance between centres of buttons.	0.18 mm. ..	0.27 mm.—0.33 mm.	0.36 mm. ..	0.58 mm.
(7) Slits in stigmal plates —				
(a) Direction of inner slits.	Dorsal and outwards	Dorsal and outwards.	Ventrally and outwards ..	Ventrally and outwards.
(b) Angle of central slits to median sagittal plane.	40°—50° ..	45° ..	Parallel ..	168°.
(c) Length of central slit.	0.25 mm.—0.3 mm.	0.2 mm.—0.25 mm.	0.36 mm. ..	0.42 mm.
(d) Number of spines on each side of central slit.	25—30 ..	22—26 ..	34—37 ..	34.
(e) Numbers of bars in central slit.	16—18. No lateral spicules.	16—20. No lateral spicules.	24—25. Lateral spicules ..	15. No lateral spicules.
(8) Post-anal ridge ..	Uncleft ..	Uncleft ..	Slightly cleft ..	Markedly cleft.
<b>B. Pupa.</b>				
(9) Length .. ..	10 mm.—12 mm. ..	8 mm.—9 mm.	13 mm. ..	8 mm.—9 mm
(10) Breadth .. ..	4 mm. ..	3.35 mm. ..	4 mm. ..	3.25 mm.

the spines and tubercles on the body; the shape and direction of the 'posterior hollow'; the size and position of the tubercles around it; and the form of the post-anal ridge.

All these points seem of importance, but whether generic or specific cannot be decided until many more species and genera are examined.

The differences given on the table below may therefore be of help to other investigators in coming to a decision as to what are the characteristics of the larvæ in the different genera and species.

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## SOME BURSATE NEMATODES FROM INDIAN AND AFRICAN ELEPHANTS.

BY

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(Received for publication, January 4, 1921.)

THIS paper deals with certain Bursate Nematodes from the Indian and African elephants, the former kindly sent me by Mr. T. Ware, Superintendent, Civil Veterinary Department, Madras; the latter collected in the Addo Bush, Cape Province, and generously placed at my disposal by Mr. H. A. Baylis. These latter had been presented to the British Museum (Natural History) by the Imperial Bureau of Entomology. All fall in the super family *Strongyloidea* of the sub-order *Strongylata*.

In the *Strongylata* Railliet and Henry, 1913 (19), the male caudal papilla, of definite number, are lengthened into rays which lift the overlying cuticle into a double funnel-shaped fold or bursa, their terminal papilla lying either upon the outer or inner surface of the bursa, not indiscriminately, but each according to regular and unvarying practice. These rays are in order the dorsal, externo-dorsal, dorso-lateral, medio-lateral, externo-lateral, latero-ventral and ventro-ventral. The papillae lie on the internal bursal surface, with the exception of the externo-dorsal and the externo-lateral. There is frequently, if not constantly, a prebursal papilla, which in the peculiar genus *Amira* (10) forms an additional bursal ray. There are two spicules, equal and similar in the species parasitizing elephants. The ova have a thin colourless shell separated by a clear space from the granular contents.

The *Strongyloidea* Lane, 1917, possess a definite pharynx and oesophagus and oral cavity. In the *Metastrongyloidea* Lane, 1917, no oesophagus or

which have been hitherto reported from elephants, this is not so. Elephants' nematodes of the super-family *Strongyloidea* fall into the families *Strongyloidea* and *Ancylostomidae*.

In the *Strongyloidea* Cobbold, 1864 (emend Lane, 1917), the cuticle forming the oral aperture is incised to form a circle of setae or denticles. The family falls into two sub-families, the *Strongylinæ*, Railliet, 1893 (sens. nov. et strict.), in which the oral capsule is more or less cup-shaped, and the *Oesophagostominae* sub-fam. nov. in which it is ring-shaped. In elephants the Strongylinæ are represented by *Equinurbia*, *Choniangium* and *Deceusia*; the *Oesophagostominae* by *Murshidia*, *Quilonia*, *Amira* and *Pteridopharynx*. The systematic position of *Strongylus foliatus* Cobbold, 1882, from the Indian elephant remains uncertain; nor can the writer agree with either Godoelst or Ihle (8), who respectively refer *Sclerostomum rectum* v. Linstow, 1907, from the African elephant to *Cylicostomum* and *Murshidia*, since he believes that on its existing description even its generic allocation is impossible. Moreover conjectures appear in this case peculiarly unprofitable, since, as already pointed out (10), Linstow, in 1906, described a *Strongylus rectus* from *Dolichotis patagonica*; so that since *Strongylus* and *Sclerostomum* are synonyms, the specific name of his elephant's nematode becomes a rejected homonym, and as such is dead beyond resurrection.

The *Ancylostomidae* Looss, 1911 (emend Lane, 1917), possess an oral aperture guarded by paired sub-ventral plates. In the *Necatorinae* Lane, 1917, these plates have unnotched curved cutting edges. In the *Necatoridi* Lane, 1917, the dorsal lobe and rays of the bursa are symmetrical as, except for minute differences, are the spicules. In elephants this tribe is represented by *Grammocephalus*, Railliet and Henry, 1910, and *Bathostomum* Railliet and Henry, 1909. With the possible exception of *Deceusia additica*, whose generic separation from *Strongylus* is perhaps not universally admitted, it is significant that the bursate nematodes of elephants are confined to genera which, so far as is known, are not represented in any other animal.

There are described below *Pteridopharynx africana*, *Quilonia africana*, *Grammocephalus clathratus*, the closely allied *Grammocephalus varedatus* and *Bathostomum saengeri*.

As regards species, the more detailed examinations hitherto carried out suggest that the same species are not found in the two hosts. It may therefore be well to reserve opinion upon the reported finding of *Murshidia falcifera* in the African elephant (21).

PLATE XI.

Figs. 1—4. *Pteridopharynx africana*.

- Fig. 1 The head from the dorsum, the oral cavity being seen in optical  
bisection, the dorsal oesophageal plume and flange from the  
outside.
- „ 2. The head from the left side, the oral cavity being seen in optical  
bisection, the dorsal oesophageal flange partly in section and  
partly in perspective. The subventral flange are not  
indicated. In figs. 1 and 2 the sessile lateral and prominent  
submedian papillae are seen a condition to be expected in the  
oesophagostomine.
- „ 3. Transverse section through the three plumes of the oesophageal  
cuticle.
- „ 4. The posterior end of the female.

# PLATE XL

Figs. 1-4. *Macropodaphysa africana*.

- Fig. 1 The head from the dorsum, the oral cavity being seen in optical dissection, the dorsal oesophageal plume and flange from the outside.
2. The head from the left side, the oral cavity being seen in optical dissection, the dorsal oesophageal flange partly in section and partly in perspective. The subventral flanges are not indicated. In figs 1 and 2 the sessile lateral and prominent submedian papillae are seen a condition to be expected in the oesophagostomium.
3. Transverse section through the three plumes of the oesophageal cuticle.
4. The posterior end of the female.

## 119



119



## PTERIDOPHARYNX \* n. sp.

Rather slender (Cesophagostominae with discoidal head (*vide* Table). The rays of the corona radiata vary in length, the dorsal and ventral being the shortest, the lateral the longest, the shape of the oral aperture being correspondingly affected. The oral cavity has the shape of an inverted tunnel, its cuticular lining coming into contact with the short, nearly circular, ring which forms the oral capsule. Its base is encroached upon by three sharp, curved, cuticular flanges, one surmounting each of the three muscular oesophageal columns (Plate XI, figs. 1 and 2). The oesophagus (Plate XII, fig. 5) is strikingly short and wide, somewhat hourglass-shaped, the central nervous system encircling the constriction, but its most characteristic feature is the plumose sculpturing of the cuticle which lines the anterior part of the internal aspect of each of its three columns. Each sculpturing has three external thickenings (Plate XI, fig. 3) running more or less antero-posteriorly and projecting into the cuticular substance of the oesophagus, the central one being the largest and most conspicuous. The free internal surface is moulded into a number of freely projecting ridges which run obliquely outwards and posteriorly, giving it the feathered appearance on which the nematode's name is based.

On the male bursa the lateral ray has a prominence or accessory ray on its posterior border, while of the three branches of each dorsal ray the two outer are fused nearly to their tips (Plate XII, figs. 6, 7 and 8). The sperules are equal and similar and an accessory piece is present.

The vulva lies immediately anterior to the anus, the vagina running anteriorly and dividing into two parallel anteriorly directed uteri, each provided with an ovjector. GENOTYPE *Pteridopharynx africana*.

## PTERIDOPHARYNX AFRICANA n.sp.

The excretory pore and cervical papillae lie posterior to the posterior end of the oesophagus (*vide* Table). These papillae are very delicate straight spines with an anterior inclination. The worm is widest midway between the anus or cloacal opening and the cephalic extremity. The oesophageal plume is 0.15 mm. long.

The buccal rays are long and slender, particularly the dorsal ones, the lateral being correspondingly shaped. The union of the two outer dorsal rays is so nearly complete that it is only on close examination that it is seen that there are more than two on each side. The sperules

\* πτερίν εϊδους φάρυγξ

166 *Bursate Nematodes from Indian and African Elephants.*

(Plate XII, figs. 9 and 10) are equal and similar each with a central rod which ends in a curve and terminates in a sharp hook and with two thin alae plumosely marked.

The vulva (Plate XI, fig. 4) opens at the summit of an anteriorly curved nipple, situated immediately anterior to the anus. The vaginal lumen expands just posterior of its middle, and after a course of 0.55 mm. divides into two parallel uteri, which also, after a course of 0.55 mm., expand into ovijectors. There were no ova in any of the females.

HOST: The African Elephant.

HABITAT: The Stomach.

NOTE.—The genus lies near *Murshidia*, being separated from it by the structure of the oesophageal cuticle, the almost complete fusion of the two outer branches of the dorsal ray, the delicate spicules, and the close approximation of anus and vulva. The intimate parallelism between Indian and African forms of *Murshidia* and *Grammocephalus* suggests that an Indian *Pteridopharynx* may yet be discovered. If 'Nematode No. 4' of Evans and Rennie do not represent *Chonangiium epistomum*, it may possibly be such a form.

QUILONIA LANE, 1914.

Synonyms. *Eicansia* Railliet and Henry, 1914, not *Eransia* Scott, 1906 (Copepod): *Nemateicansia* Ihle, 1919.

Fairly slender oesophagostominæ with discoidal head, the rays of the corona radiata being of nearly equal length and separated from the rest of the head by a trench. The cuticle lining the oral cavity is markedly disassociated from the ring-shaped oral capsule. Each of the subventral oesophageal columns bears on its apex a tooth.

In the bursa each dorsal ray is tridigitate, the cleft between the outer and middle being deeper than that between the middle and inner branches. Since the dorsal rays are long the bursa is correspondingly elongated. The spicules are equal and similar with sickle-shaped points. The accessory piece arches round the spicular canal, the ventral edge being prolonged at one part into a point.

The vulva marked by adherent dark cement, lies in the third quarter; the short centripetally-running vagina divides at once into two opposed uteri bearing marked ovijectors, the posterior uterus immediately curving anteriorly.

Genotype. *Quilonia renniei* Railliet and Henry, 1914, from the Indian elephant.

Other Spec.es. *Quilonia travancra* Lane, 1914, from the Indian elephant; and *Quilonia apiensis* Geddoelst, 1916, and *Quilonia africana* n. sp., both from the African elephant.

PLATE XII

Figs. 5--10. *Pseudopharyngodon*.

- Fig. 5. The anterior end from the dorsum showing the most anterior  
oesophagus.  
" 6. The bursa from the right side.  
" 7. The bursa from the dorsum.  
" 8. The tip of the outer branch of the dorsal ray.  
" 9. The scapula and accessory piece from the dorsum. The median  
sheath surrounding the anterior part of the organ is  
indicated.  
" 10. The tip of aspicule.

# PLATE VII.

Fig. 7—10. *Macrobathrus africanus*

- |      |     |                                                                                                                                         |
|------|-----|-----------------------------------------------------------------------------------------------------------------------------------------|
| Fig. | 7.  | The anterior end from the dorsum showing the short powerful oesophagus.                                                                 |
| "    | 8.  | The bursa from the right side.                                                                                                          |
| "    | 9.  | The bursa from the dorsum.                                                                                                              |
| "    | 10. | The tip of the outer branch of the dorsal ray.                                                                                          |
| "    | 11. | The spicules and accessory pieces from the dorsum. The spicular sheath surrounding the anterior part of the right spicule is indicated. |
| "    | 12. | The tip of a spicule.                                                                                                                   |

PLATE XII

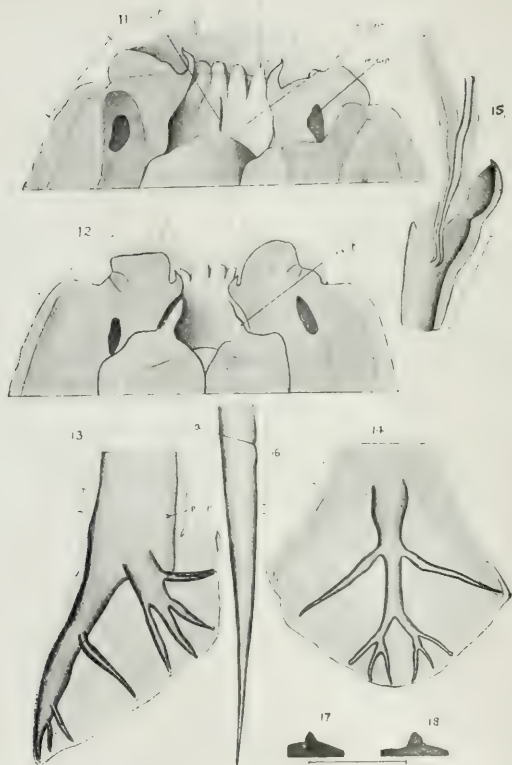


CLAYTON LANE.—Some Bursate Nematodes from Indian and African Elephants.





PLATE XIII.



CLAYTON LANE.—Some Bursate Nematodes from Indian and African Elephants.

# Table 2/III

Table 2/III - (continued)

11	The point from the 1st and 2nd optical position.	10
12	The point from the 3rd and 4th optical position.	10
13	The point from the 5th and 6th optical position.	10
14	The point from the 7th and 8th optical position.	10
15	The point from the 9th and 10th optical position.	10
16	The point from the 11th and 12th optical position.	10
17	The point from the 13th and 14th optical position.	10
18	The point from the 15th and 16th optical position.	10
19	The point from the 17th and 18th optical position.	10
20	The point from the 19th and 20th optical position.	10
21	The point from the 21st and 22nd optical position.	10
22	The point from the 23rd and 24th optical position.	10
23	The point from the 25th and 26th optical position.	10
24	The point from the 27th and 28th optical position.	10
25	The point from the 29th and 30th optical position.	10
26	The point from the 31st and 32nd optical position.	10
27	The point from the 33rd and 34th optical position.	10
28	The point from the 35th and 36th optical position.	10
29	The point from the 37th and 38th optical position.	10
30	The point from the 39th and 40th optical position.	10
31	The point from the 41st and 42nd optical position.	10
32	The point from the 43rd and 44th optical position.	10
33	The point from the 45th and 46th optical position.	10
34	The point from the 47th and 48th optical position.	10
35	The point from the 49th and 50th optical position.	10
36	The point from the 51st and 52nd optical position.	10
37	The point from the 53rd and 54th optical position.	10
38	The point from the 55th and 56th optical position.	10
39	The point from the 57th and 58th optical position.	10
40	The point from the 59th and 60th optical position.	10
41	The point from the 61st and 62nd optical position.	10
42	The point from the 63rd and 64th optical position.	10
43	The point from the 65th and 66th optical position.	10
44	The point from the 67th and 68th optical position.	10
45	The point from the 69th and 70th optical position.	10
46	The point from the 71st and 72nd optical position.	10
47	The point from the 73rd and 74th optical position.	10
48	The point from the 75th and 76th optical position.	10
49	The point from the 77th and 78th optical position.	10
50	The point from the 79th and 80th optical position.	10
51	The point from the 81st and 82nd optical position.	10
52	The point from the 83rd and 84th optical position.	10
53	The point from the 85th and 86th optical position.	10
54	The point from the 87th and 88th optical position.	10
55	The point from the 89th and 90th optical position.	10
56	The point from the 91st and 92nd optical position.	10
57	The point from the 93rd and 94th optical position.	10
58	The point from the 95th and 96th optical position.	10
59	The point from the 97th and 98th optical position.	10
60	The point from the 99th and 100th optical position.	10

PLATE XIII.

Figs. 11-16. *Quilonia africana*.

- Fig. 11. The head from the left side in optical bisection.  
„ 12. The head from the venter in optical bisection.  
„ 13. The bursa from the right side.  
„ 14. The bursa from the dorsum.  
„ 15. The accessory piece in optical bisection, and the tips of the  
spicules, from the left side.  
„ 16. The posterior end of the female from the right side.  
„ 17. *Quilonia remiei*. The subventral tooth.  
„ 18. *Quilonia travancra*. The subventral tooth.

## QUILONIA AFRICANA n. sp.

The sunken corona consists of ten massive rays (Plate XIII, figs. 11 and 12). The subventral teeth are long and sharp, more so than in the Indian species (cf. Plate XIII, figs. 17 and 18). The excretory pore and cervical papillae lie about the posterior end of the oesophagus (*vide* Table). The greatest breadth in both sexes is situated about midway between the anus or cloacal opening and the anterior extremity.

The bursal rays (Plate XIII, figs. 13 and 14) are fairly delicate. The cleft between the medio-lateral and dorso-lateral rays is deeper than that between the medio-lateral and externo-lateral. The spicules (Plate XIII, fig. 15) have fine shafts and delicate striated alae. The large accessory piece is very complete laterally and its ventral point is incurved.

The tail of the female is exceptionally long and the point fine even under high powers.

HOST: The African Elephant.

HABITAT: The Stomach.

NOTE.—Since differences in form, more markedly than in measurement, distinguish the congeneric species of elephant's nematodes, it is unfortunate that circumstances compelled Gschelst to publish unillustrated his description of *Quilonia apicatus*. The number of rays in the corona, the position of the excretory pore and the length of the female's tail suffice to separate the two African forms. The presence of the marked subventral teeth of *Quilonia africana* has led to a re-examination of the two Indian forms, and small teeth have been detected in them also (in Plate XIII, figs. 17 and 18). The corona in *Quilonia africana* is sunken as it is in *Quilonia trivancora*. Its position in *Quilonia apiensis* is unnoted.

The writer has found *Quilonia trivancora* in copula. The position adopted was unexpected. The dorsal ray of the bursa was directed towards the head of the female, and the two worms lay parallel with their heads in opposite directions. Presumably this characteristic is generic.

## GRAMMOCEPHALUS RAILLIET AND HENRY, 1910.

Necatoridi having the head curved towards the dorsum and the mouth directed obliquely, dorsally and anteriorly. The mouth capsule is wide anteriorly and narrowed posteriorly, the narrowing being abrupt on the dorsal wall and accompanied by an infolding of the capsule. Posterior of the fold lie a median dorsal tooth, given out to the distal of the oesophageal gland, and a triangular pair of lateral and of subventral teeth, which are accordingly more anteriorly situated than is usual. The subventral teeth have the free apex replaced by a notch, and the lateral have the anterior surface concave, so that the free apex forms

an acute angle. The œsophagus is long and simple. From the dorsal wall of the intestine, close to its union with the œsophagus, springs a long diverticulum, flattened dorso-ventrally (Plate XV, fig. 24), reaching to about the level of the excretory pore. There is a pair of cephalic glands nearly as long as the œsophagus, whose posterior portions are packed between the œsophagus and the intestinal diverticulum. The cervical glands are small.

Of the bursal rays (Plates XII, XIV, figs. 10 and 22) the externo-lateral curves ventrally, the medio-lateral and postero-lateral dorsally. The dorsal rays (Plate XV, fig. 26) are united from their base to the point of origin of the externo-dorsal rays, separate beyond, their extremities are bilid, the outer branch curving, the inner inclining towards the midline. The spicules (Plate XV, fig. 27) are strong with a massive central thickening and an ala on each side, that towards the midline being the larger and marked by a fine etching of delicate, dichotomously dendritic lines. There is no accessory piece.

The vulva lies close to the equator. The centripetally running and very short vagina ends almost immediately in the two divergent ovijectors. These are probably family characteristics.

Genotype. *Grammocephalus clathratus*.

GRAMMOCEPHALUS CLATHRATUS (Baird, 1868). Railliet and Henry, 1910.

Synonyms. *Sclerostoma clathratum* (Baird, 1868); *Strongylus clathratus* Cobbold, 1882.

The semilunes (Plates XIV, XV, figs. 19 and 25) are large, the fold in the dorsal wall of the oral capsule marked, the median dorsal tooth not particularly prominent, the apical notches in the subventral teeth large, the anterior edges of these teeth face anteriorly and ventrally, the anterior edges of the lateral teeth lie well posterior of the corresponding edges of the subventral teeth. The intestinal diverticulum measures 2.3 mm. (For other measurements *vide* Table.)

The bursal rays (Plate XIV, fig. 20) are relatively long. The body of the female narrows abruptly about the anus and the first part of the tail is nearly cylindrical (Plate XV, fig. 23).

HOST: The African Elephant.

HABITAT: Bile ducts.

GRAMMOCEPHALUS VAREDATUS n. sp.

Synonyms. 'Nematode No 1' Evans and Rennie, 1910; *Grammocephalus clathratus* (Baird) Railliet and Henry, 1910. In *Grammocephalus*

PLATE XIV.

Figs. 19 and 20. *Grammocephalus clatiratus*.

- Fig. 19. Optical bisection of the head from the right side.  
" 20. The bursa from the left side.

Figs. 21—22. *Grammocephalus caradatus*.

- Fig. 21. Optical bisection of the head from the left side.  
" 22. The bursa from the right side.

PLATE XIV.

- Fig. 19 and 20. *Commocarpus chalybeatus*.  
 19. Optical dissection of the head in the right side.  
 20. The bases from the left side.  
 Fig. 21—22. *Commocarpus canadensis*.  
 21. Optical dissection of the head in the left side.  
 22. The bases from the right side.

PLATE XIV.

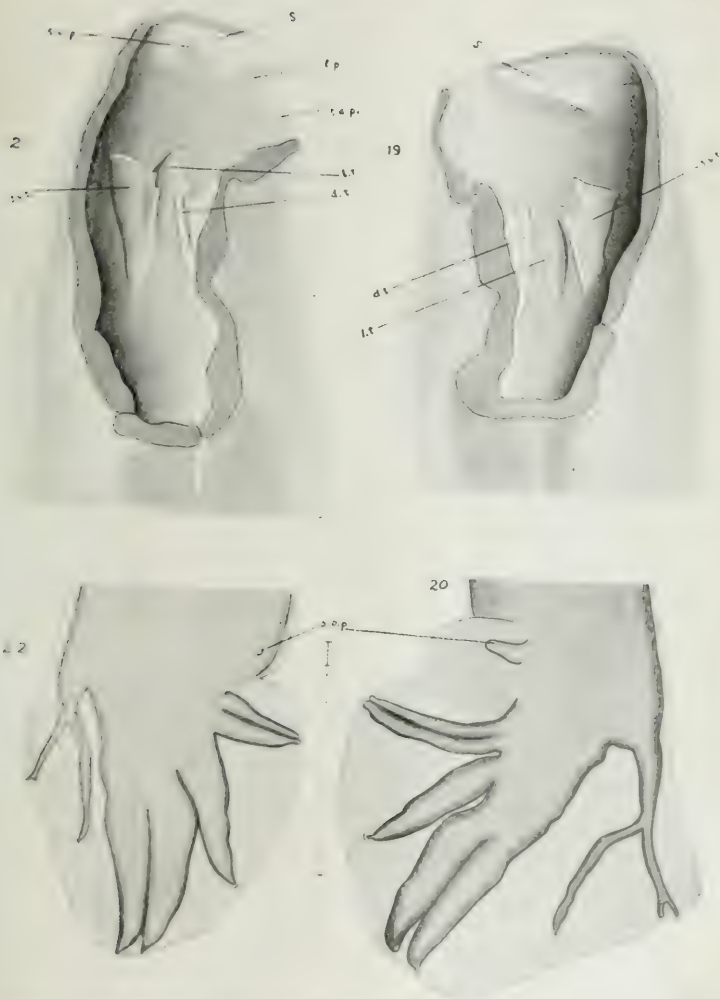
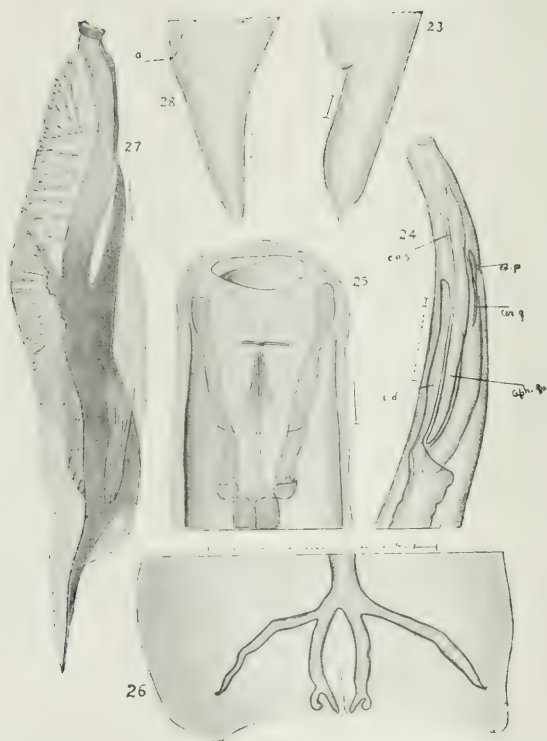






PLATE XV.



CLAYTON LANE.—Some Bursate Nematodes from Indian and African Elephants.

VZ. '34A.1'

- [illegible]

PLATE XV.

Figs. 23-26. *Grammnocephalus clathratus*.

- Fig. 23. The posterior end of the female from the left side  
.. 24. The head end from the right side.  
.. 25. The head end from the dorsum.  
.. 26. The bursa from the dorsum.

Figs. 27 and 28. *Grammnocephalus raredatus*

- Fig. 27. The right spicule from the dorsum.  
.. 28. The posterior end of the female from the left side.

*crenulatus* \* the semilunes (Plate XIV, fig. 21) are somewhat small, the fold in the dorsal wall of the oral capsule is not markedly conspicuous, the ventral projection of the dorsal tooth is considerable, the apical notches of the sub ventral teeth are small and the anterior edges of these teeth face anteriorly and dorsally, the anterior edges of the lateral teeth do not lie posterior of the corresponding edges of the sub-ventral teeth. The intestinal caecum measures 2.2 mm. For other measurements *vide* Table.

The bursal rays are relatively short (Plate XIV, fig. 22).

The tail of the female is conical (Plate XV, fig. 28).

HOST: The Indian Elephant.

HABITAT. Bile ducts.

NOTE.—Baird's original material of *Graemiocephalus clathratus* (2) consists of a single male of the species here described as *Graemiocephalus clathratus*, and of males and females of a species belonging to a widely different family. Baylis (3) has explained the good reasons for designating as *Graemiocephalus clathratus* Baird's single male type specimen. C. de Meillon's perplexity in this matter (5) is now readily understandable. The African material now examined, although the males are smaller than is Baird's specimen, unquestionably belongs to the same species.

The genus is of marked interest in several directions. The presence of an intestinal diverticulum, a fact noted by Evans and Rennie but since disregarded, appears to be unique among the Strongylata; the anterior position of the teeth, usually found at the base of the oral cavity, is at least exceptional; the females in the African and Indian material examined as well as in that reported upon by Evans and Rennie, are smaller than the males; the differences between the two species are differences in shape, and the ordinary numerical and unillustrated description would, and indeed has, entirely overlooked them.

#### BATHYSTOMUM RAILLET and HENRY, 1909

Small Necatoridi having the head curved towards the dorsum (Plate XVI, fig. 29), the oral aperture facing anteriorly and dorsally. The oral capsule is fissured on its dorsal lateral aspects, and its internal surface is raised mainly ventrally and laterally into shelf-like projections encroaching on the oral cavity. A small dorsal tooth surmounts the dorsal column of the oesophagus, which is simple and cup-shaped.

In the male bursa the dorsal rays are separate for nearly their whole extent (Plate XVI, fig. 31), the external dorsal rays springing from the individual dorsal rays. The lateral rays (Plate XVI,

\* Nomenclature acknowledged of my indebtedness to Mr. S. W. Baylis.

fig. 33) have a ventral direction. The spicules are stout. There is no accessory piece.

The vulva lies near the equator, the short centripetal vagina dividing immediately into the two opposed uteri furnished with strong ovijectors.

Genotype. *Bathmostomum sangeri*.

BATHMOSTOMUM SANGERI (Cobbold, 1882) Railliet and Henry, 1909.

Synonyms. *Doehmius sangeri* Cobbold, 1882; *Uncinaria os-pillatum* Piana and Stazzi, 1900.

The semilunes are large and massive (Plate XVI, figs. 31 and 32). Owing to the unsatisfactory state of preservation of the material the worms would not roll, and correlation of the structures seen in lateral and ventral views of the oral cavity was imperfectly attained. The most anterior shelf is limited to the lateral face of the oral cavity. The next, either single or divided, passes across its ventral face from one side to the other. At the base of the oral cavity there appears to be a pair of subventral teeth, in connection with which a complete shelf appears to encircle the whole base of the oral cavity. The small dorsal tooth gives, as usual, exit to the oesophageal duct. The oral cavity is wider than long. The stout cervical papillæ curve posteriorly (Plate XVI, fig. 30).

The bursal rays are fairly stout, the lateral and ventral rays tapering steadily; the divisions between the three lateral rays are of about the same depth; the prebursal papilla is large. The stoutish spicules (Plate XVI, fig. 35) are thickened, first dorsally, then ventrally, the latter portion passing on into the very sharp, truncated point. The cement gland measures 0.27, and the kidney-shaped vesicula seminalis 0.06 mm.

The tail of the female forms a long cone, narrowing abruptly near the point, which however is itself prolonged.

HOST: The Indian Elephant.

HABITAT: Caecum.

NOTE. —The state of preservation prevented the possibility of recognising the presence or absence of cephalic and cervical glands; but since it is a genotype, and because of the parallelism between Indian and African forms, it is felt that even a partial description may be of value.

The steady destruction of the African elephant now proceeding renders a fuller examination of its parasites a matter of extreme zoological interest and importance. Even of greater interest and value would be

PLATE XVI.

Figs. 29-37. *Bathmostomum saundersi*.

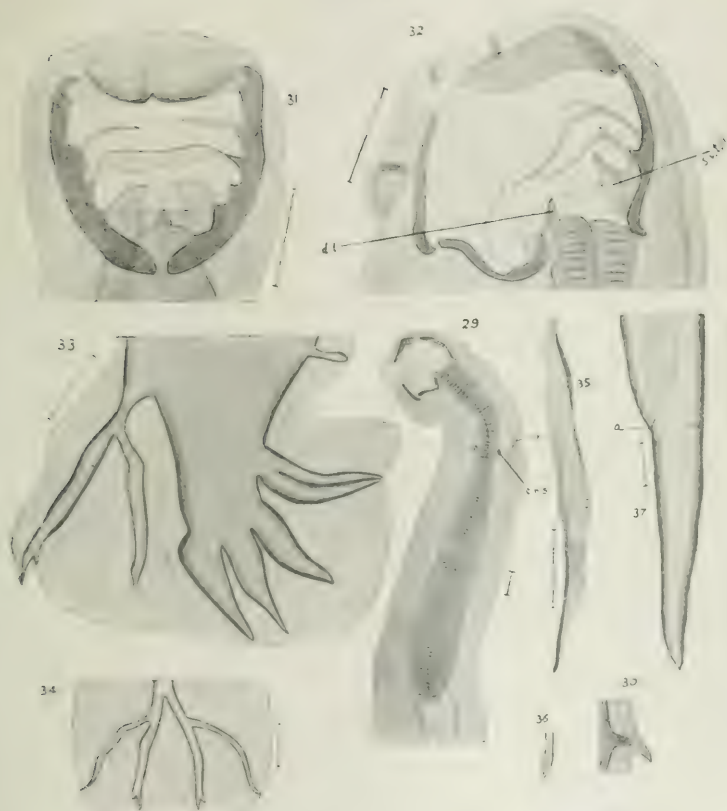
- Fig. 29. The head end from the right side.  
30. A cervical papilla.  
31. The head end from the venter.  
32. The head end from the right side. The three sessile cephalic papillæ are shown. They appear to be characteristic of the family.  
33. The bursa from the right side. Note the large prebursal papilla.  
34. The bursa from the dorsum.  
35. A spicule.  
36. The tip of a spicule more highly magnified.  
37. The posterior end of the female from the left side.

PLATE XVII.

Figs. 29-37. *Halimolobos* (various).

- |      |     |                                                                                                                                  |
|------|-----|----------------------------------------------------------------------------------------------------------------------------------|
| Fig. | 29. | The head end from the right side.                                                                                                |
| "    | 30. | A cervical papilla.                                                                                                              |
| "    | 31. | The head end from the center.                                                                                                    |
| "    | 32. | The head end from the right side. The three sessile cephalic papillae are shown. They appear to be characteristic of the family. |
| "    | 33. | The bases from the right side. Note the large prebuccal papilla.                                                                 |
| "    | 34. | The bases from the dorsum.                                                                                                       |
| "    | 35. | A spine.                                                                                                                         |
| "    | 36. | The tip of a spine more highly magnified.                                                                                        |
| "    | 37. | The posterior end of a female from the left side.                                                                                |

PLATE XVI.



CLAYTON LANE.—Some Bursate Nematodes from Indian and African Elephants.





172 *Bursate Nematodes from Indian and African Elephants.*

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# LETTERING AND SCALES.

a.	..	..	..	Anus.
a.p.	..	..	..	Accessory piece.
ceph. g.	..	..	..	Cephalic gland.
cer. g.	..	..	..	Cervical gland.
cn.s.	..	..	..	Central nervous system.
c.r.	..	..	..	Corona radiata.
d.	..	..	..	Dorsal ray.
d.l.	..	..	..	Dorso-lateral ray.
d.t.	..	..	..	Dorsal tooth.
ed.	..	..	..	Extensio-dorsal ray.
ex.p.	..	..	..	Excretory pore.
i.d.	..	..	..	Intestinal diverticulum.
l.p.	..	..	..	Lateral papilla.
l.t.	..	..	..	Lateral tooth.
l.v.	..	..	..	Latero-ventral ray.
m.l.	..	..	..	Medio-lateral ray.
oes.	..	..	..	Oesophagus.
oes.f.	..	..	..	Oesophageal flange.
oes.p.	..	..	..	Oesophageal plume.
or. cap.	..	..	..	Oral capsule.
or. cav.	..	..	..	Oral cavity.
ovej.	..	..	..	Ovijector.
p.b.p.	..	..	..	Prebursal papilla.
s.	..	..	..	Semilune.
s.p.	..	..	..	Spicule.
s.v.p.	..	..	..	Subventral papilla.
s.v.t.	..	..	..	Subventral tooth.
vu.	..	..	..	Vulva.
v.v.	..	..	..	Ventro-ventral ray.

All scales in uninterrupted lines represent 0·1 mm. Where there is a dotted extension, the total scale, continuous and interrupted, represents 1·0 mm.



# PHYSIOLOGICAL CHANGES AT HIGH ALTITUDES AND THEIR RELATION TO MOUNTAIN-SICKNESS. \*

BY

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(Received for publication, January 11, 1921.)

THIS paper is the result of observations made at high altitudes in the Western Himalaya and on the Pamir Plateau while serving with an expedition despatched by the Survey of India to complete the link between the triangulations of India and Russia. I think the subject may be worthy of record, as I can find no account of any similar series of observations made over so long a period at so considerable a height.

## GENERAL REMARKS.

It is a very remarkable fact that a man can make an ascent to a height of 18,000 or 20,000 feet and suffer a bodily inconvenience so slight that some have scarcely observed it. At sea-level there is an atmospheric pressure on the surface of the body amounting to 15 pounds on every square inch. If the surface area of the body be taken as 2,000 square inches then the total weight supported by the body at sea level will amount to about  $13\frac{1}{2}$  tons. A man commences to ascend. For every 1,132 feet he rises the barometer falls one inch and the load on his body is reduced by about 1,000 pounds. When he reaches 10,000 feet the weight that presses on him is diminished by some four tons and at 15,000 feet he is relieved of a pressure equal to a weight of six tons. How great is this change and how little are we aware of its occurrence! The pressure has diminished from  $13\frac{1}{2}$  tons to 7 tons and we

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\* For presentation as a thesis for the degree of M. D.

scarcely know it. There may be some palpitation on exertion, some difficulty in breathing, possibly a throbbing headache and a sense of undue fatigue, but the healthy man may find little else to make him aware that anything unusual has occurred. His mind and body remain active, he can still perform his strenuous duties quite oblivious of the great pressure from which his body has been relieved.

Nevertheless, it is a certain fact that in any continued ascent an altitude will always be reached when the body vigour begins to decline and those distinct feelings of embarrassment ensue which we are accustomed to call mountain-sickness. It would seem impossible to define in terms of feet exactly the altitude where the symptoms first appear. Some persons are affected at a lower, others at a higher, altitude, some who escape in fine weather, are attacked in storm and fog, some who appear free in the mountains of one country, easily succumb elsewhere. Indeed, there are men of experience who are prepared to deny that any such malady occurs at all. Mountaineers of the highest authority have in one place met with the discomfort as low as 7,000 feet and in other places scarcely observed it at 17,000 feet. The brothers Schlagintweit in their journeys through Thibet felt nothing more than acute fatigue at an altitude of 22,230 feet, and the party led by the Duke of the Abruzzi to explore the Karakoram suffered little inconvenience at the great height of 24,600 feet.

#### RECORDED SYMPTOMS.

A condition so variable in its appearance is equally capricious in its mode of attack. Most travellers complain that, when a certain altitude is reached, they find that they are enduring a discomfort which increases at every step, that a feeling of oppression weighs down the chest, that they pant and blow to an inordinate degree and feel the heart beating within the chest, that with every exertion these inconveniences recur, and after a short rest quickly pass away. These are the usual discomforts which appear to embarrass all. But there are other symptoms more variable in their appearance which sometimes add to the general distress. The stomach is often attacked. Men may lose their appetites, suffer acute cramps in the abdomen, find themselves nauseated or actually vomit. The functions of the brain are frequently involved. The head throbs with a sense of pain at every beat, complaints have been made of a feeling of drowsiness, a giddiness, a loss of mental power, a mind becoming more torpid with will and memory failing, even an exhaustion that sinks

into unconsciousness. Bleedings from the mucous membranes are well known, but not very usual manifestations. The blood may flow from the gums, the lips, more often from the nose, or the conjunctivæ may become engorged and the blood trickle down the cheeks. Nor do the special senses always escape. Mountaineers have complained of buzzing in the ears and signs of incipient deafness, some have noticed peculiar changes in the sense of taste, others an increased sensitiveness to odours, others a recognition of failing vision. Over all there hangs the burden of exceptional fatigue. The heart is beating at an excessive rate, the breathing is deep and laboured, the skin is sometimes livid, yet the traveller struggles on. He is unable to perform the same work with the usual amount of effort, every exertion exaggerates the ill-effects, and he finds himself involved in a bodily distress such as he never experienced at sea-level.

As for myself, I noticed the first signs of the inconvenience at an altitude of 11,000 feet after a rapid ascent from a height of 5,000 feet. There I was attacked with those feelings of discomfort that have so often been described. I felt a sensation of exhaustion out of all proportion to the toil of the ascent. The breathing became laboured, the inspirations deeper and the pulse rate markedly increased. A throbbing headache developed, becoming more acute at every exertion. With this was accompanied a feeling of giddiness especially on attempting to move after sitting down to a short rest. These are the first warnings that give notice of any change. The gastric and other troubles follow at higher altitudes. They scarcely affected us below a height of 17,000 feet. All people suffer with different degrees of intensity. One man is confident that he feels no embarrassment, another, like Father de Acosta in Peru, declares that 'I was surprised with such pangs of straining and casting as I thought to cast up my soul too, for having cast up meate, fleugme, and choller, both yellow and green, in the end I cast up blood with the straining of my stomach.....I therefore persuade myself that the element of the air is there so subtile and delicate as is not proportionable with the breathing of man, which requires a more gross and temperate air, and I believe it is the cause that does so much aiter the stomach and trouble all the disposition.'

#### VIEWS AS TO CAUSATION.

The question obviously arises, as it did to Father de Acosta, *what is the cause of this disturbance in the bodily mechanism that occurs during*

an ascent? Many answers, some founded on scientific observation, others based on the mere fancy, have been given to this question. The eminent physiologist, Paul Bert, whose study of the subject met with wide acceptance, considered it to be a simple asphyxia the consequence of the diminished supply of oxygen available at great heights. And this also is the view of no less an authority than Sir Clifford Allbutt. Others have sought for the explanation not so much in the diminution of oxygen as in the reduction in the atmospheric pressure. Then there are many theories in which different organs of the body are believed to be specially involved and to account for the general disturbance. Some have attributed the complaint to the excessive work of the muscles and have endeavoured to explain all on a theory of mere fatigue. But this I feel sure is untrue, for Mr. Whymper did not escape mountain-sickness when he rode on horseback to an elevation of 16,500 feet, and we suffered from distinct symptoms when riding to 17,000 feet on yaks. To others a disordered state of the digestive functions has seemed a sufficient cause to account for the whole train of symptoms. It has been thought that a weakness of the heart's action following the severe exertion would explain the nature of the attack. Other views turned to the lungs as the seat of the trouble. It was imagined that those who suffered had a pulmonary capacity below the average, but this surmise was easily disproved. Then changes were believed to occur in certain organs as a consequence of the reduction in the atmospheric pressure. The blood, it was said, might accumulate in the lungs and obstruct the breathing. Gaseous matter, under the diminished external pressure, might distend the abdominal cavity and interfere with the heart's action, or even gas might effervesce in the blood stream. From such changes it was thought severe consequences would follow sufficient to give rise to mountain-sickness. Other investigators sought for a cause in some disturbance of the nervous system. Angelo Mosso, after a prolonged enquiry, came to the conclusion that a depression of the nerve centres was the most probable cause. A change in the circulation of the blood through the brain has also been advanced as an explanation; the conflicting views of an anæmia and a congestion having both received support. Others have laid more stress on psychological influences and have looked to the emotions, anxieties and apprehensions of the mountaineer for the origin of his distress. Then there are the common discomforts associated with the ascent which, if not the exciting cause must contribute in some degree to the general disablement. There is the cold, the glare, the extreme effort, the physical fatigue, perhaps the

unusual privations, the insufficient sleep, the unsuitable and unaccustomed food. Fanciful ideas have also had a place in the many attempts at an explanation. Some have looked to a vague mysterious quality in the atmosphere, others to an exhalation from noxious mountain herbs.

But the cause to which I wish to direct attention is that which depends on the increase in the number of red corpuscles that occurs during an ascent to any considerable height.

#### INCREASE IN THE NUMBER OF RED CORPUSCLES DURING AN ASCENT TO A HIGH ALTITUDE.

At sea-level the percentage of oxygen consists of 20.96 of any volume of air. About one-fifth of the air breathed is thus pure oxygen. A man ascends. He reaches 7,000 feet. The pressure is here reduced by about one-quarter, so that the supply of oxygen is only three-quarters of that available at sea-level. He continues the ascent. He reaches a height of 18,500 feet and finds that the available supply of oxygen is halved. Could he ascend further and reach the summit of Everest, a height of 29,000 feet, he would have at his disposal only one-third of the supply of oxygen that he was accustomed to use at sea-level.

Man is suitably adjusted to his environment. At sea-level he has 5,000,000 red corpuscles in every cubic millimetre of blood, and this number is sufficient to deal with the oxygen available at that level. But when an ascent is made and the supply of oxygen is diminished in amount, then the 5,000,000 corpuscles which served the purpose at sea-level are insufficient at the greater height. Something must occur in order to restore the balance. A change does occur. The body manufactures more corpuscles. This is the method by which it adjusts itself if it is given sufficient time. When the man reaches 8,000 feet the number of his corpuscles has risen to about 6,000,000, when he reaches 13,000 feet the corpuscles have risen to 7,500,000 and at 18,000 feet the number will probably exceed 8,000,000 per cubic millimetre of blood.

I will enter into a little detail on this physiological change in the constitution of the blood as I think it explains the essential facts connected with mountain-sickness. I made many observations on the blood of different people while ascending to the Pamir Plateau, while resident on the plateau and also during the descent.

I come first to the changes that took place in the blood of one person during the ascent. The upward progress from a lower to a higher altitude was on the whole very gradual. Of course at intervals we had to cross comparatively high passes and to sink into low-lying troughs between the ranges. These occurrences would tend in some degree to interfere with the results, but, considered broadly, the ascent was extremely gradual, so much so that nearly two months were occupied in passing from the lowest point at 700 feet to the highest point reached, 18,203 feet.

The changes that occurred in this gradual ascent I tabulate as follows:—

Date.	Altitude.	Corpuscles per cu. mm.
April 10th	700 feet	4,880,000
May 12th	4,390 "	5,240,000
May 21st	8,000 "	6,040,000
May 28th	10,000 "	6,624,000
May 30th	11,060 "	6,760,000
June 1st	12,100 "	6,800,000
June 21st	13,300 "	7,525,000
June 23rd	15,600 "	7,840,000
June 26th	16,907 "	7,640,000
July 27th	18,203 "	8,320,000

It is clear from the above table that for every foot in a gradual ascent there is a continuous and fairly regular increase in the number of red corpuscles per unit volume of blood. It will be noticed that at 700 feet the blood was somewhat below the normal standard of richness and at 18,000 feet the number had become almost doubled. The rate of increase is rapid, it can be observed from day to day. Thus the following observations indicate how the number of corpuscles increases when an ascent of about 600 feet is made on four successive days:

Date.	Altitude.	Corpuscles per cu. mm.
May 8th	5,300 feet	5,200,000
May 9th	5,900 "	5,920,000
May 10th	6,500 "	6,000,000
May 11th	6,200 "	5,840,000

This last table is not so satisfactory since for some unknown reason, a diminution was observed at 6,200 feet.

The numbers in both tables are however sufficient to indicate that an ascent from a low altitude to a high altitude is associated with a definite and continuous change in the constitution of the blood. The red corpuscles are increased in number in order to deal with the diminished supply of oxygen available at the greater height. And this is the mechanism by which acclimatisation takes place.

I may mention here that certain objections have been taken to the conclusion that an increase in the number of corpuscles occurs during an ascent. It has been said that this increase is apparent but not real. The corpuscles only appear to have undergone an increase owing to some change in the distribution of the elements of the blood. More of the corpuscles may have accumulated in the superficial vessels and a corresponding diminution would be found in the deeper organs. Or it has been thought that the blood becomes more concentrated owing to the greater excretion of water at these heights, and that this would account for the apparent increase. But these views have not gained wide acceptance. They are not easy to reconcile with the gradual growth of the corpuscles and their appearance only during a prolonged ascent. Moreover, if the change was merely a relative one it is not likely that it would be permanently established, as I will show later, in those who live their whole lives at very considerable heights. The change is something real. It is a definite addition to the number of red corpuscles that circulate in the blood stream.

#### RELATION OF CORPUSCULAR INCREASE TO MOUNTAIN-SICKNESS.

Now this increase in the number of corpuscles has a distinct relation to mountain-sickness. The development of the corpuscles is a gradual process. If a man ascends in one day from sea-level to say 12,000 feet, he certainly has not at the end of that day increased the richness of his blood to the extent of some  $1\frac{1}{2}$  million corpuscles for every cubic millimetre. There has not been time for that increase to occur, yet, until it has taken place, the man has not adapted himself to his new surroundings. He is not acclimatised. The blood manufacturing powers of his body are hard at work, but until they have produced the additional  $1\frac{1}{2}$  million required at that height he is not compensated to the new conditions. He is comparatively anæmic and suffers from those feelings of incapacity which we are accustomed to call mountain-sickness.

He suffers from a breathlessness and palpitation because his lungs and heart have to act with increased force until new corpuscles are

developed in sufficient numbers to suit the rarified air. He complains of headache, giddiness, faintness, nausea, insomnia, possibly a diminished mental power or some failure of the special senses, all of which are the symptoms of the comparative anæmia which has not yet been made good. He is mountain-sick because he is not yet acclimatised. But the disability is only temporary. The body has not yet adjusted itself to the new conditions. Acclimatisation, though in progress, is not yet complete.

#### ACCLIMATISATION TO GREAT HEIGHTS.

But acclimatisation will in time occur. If the man continues to remain at 12,000 feet the distress from which he suffers will gradually pass away, and in a few weeks he will find himself as comfortable as he was at sea-level. He is no longer mountain-sick because he has become acclimatised. This, however, has been doubted by some authorities. Sir Clifford Allbutt considers that 'even the hardened mountaineer does not lose the true mountain-sickness by habituation.' Nevertheless I believe that acclimatisation is real. It occurs everywhere. Whymper recognised it in the Equatorial Andes and the Schlagintweits in the mountains of Thibet. I will show that man becomes habituated to low pressures on the Pamir also.

The altitude of the Pamir valleys is on an average about 13,500 feet. On first ascending to these valleys many of our party suffered from the usual embarrassment associated with the height. As for myself I had a throbbing headache, a difficulty in breathing on slight exertion, a sleeplessness at night and a feeling of giddiness especially when rising from the ground. Others were less fortunate. Two men were attacked with vomiting and fever and were so prostrated as to be unable to work. After a residence of three days at this altitude, our discomforts had appreciably diminished and after a week we felt little more distress than if we were at sea-level. Acclimatisation had taken place. This was not only evident from our sensations but was more definitely settled by an examination of the blood. The number of corpuscles in my blood at 700 feet amounted to 4,480,000 per cu. mm., but the mean of a number of examinations made on the Pamir valleys over a period of two months showed that the number had increased to 7,402,000. This was the test of acclimatisation, and when this number was reached the symptoms of mountain-sickness disappeared.

Different peoples are affected in the same way, and the following are the averages of a series of counts made on three types of people who ascended in our party to the Pamir.

Race.	Corpuscles per cu. mm.
Englishman . .	7,402,000
Kashmiri . .	7,500,000
Gurkha . .	7,140,000

Thus it is clear that a residence in high altitudes results in acclimatisation, and that the method of acclimatisation is by the development of more red blood corpuscles.

Our work often took us from the valleys to the summits of the ranges that rise from the plateau to a height of from 17,000 to 18,000 feet. At these altitudes we again experienced discomfort. We were acclimatised to 13,000 feet but not to a still greater altitude. At 18,000 feet we again felt to some degree disabled. Our breathlessness, dyspepsia, and diminution of bodily vigour all tended to reappear. But just as acclimatisation followed on a residence at 13,000 feet, so it also developed after a stay at greater heights. We remained for nine days on the summit of a pass at 15,600 feet and, as a consequence, the blood count had risen to 7,840,000. After a stay of two days at 16,937 feet the count was 7,640,000, and after a similar length of stay at 18,203 feet the number of corpuscles had reached the highest I have observed, *viz.*, a count of 8,320,000. We remained at this height for only two days. The time was too short for complete acclimatisation to take place, but the count of 8,320,000 indicates that the manufactory of new corpuscles was working at high pressure and that acclimatisation would soon take place.

That acclimatisation is definitely associated with an increase in the number of the red corpuscles is made evident in another way. We had in our party one servant, who remained persistently ill for days after he had ascended to the plateau. When every body else had become acclimatised he still suffered from a severe distress. His appearance remained distinctly livid and he complained of headache and dyspnoea. On the slightest exertion his breathing became laboured and he was seized with attacks of vomiting and fever. This soon failed to adapt himself to the new conditions and he was quite unable to work. The

cause of his failure in adaptation was evident from the examination of his blood. The number of his corpuscles had reached only 5,760,000 while the blood-count normal for that altitude was over 7,000,000. For some reason the manufactory of blood corpuscles had in this man partially broken down and he failed to become acclimatised. His condition very slowly improved. After two months of residence at 13,500 feet, his blood-count had increased to 7,720,000. He was then acclimatised and all his symptoms disappeared.

The question arises, to what degree can acclimatisation take place? We can gain scarcely any information from experiments made in pneumatic chambers or from the result of observations made in aeroplanes and balloons. Acclimatisation is so gradual a process that we must rely on prolonged observations made on solid ground. From these I am confident that a man can easily adapt himself to a height of 13,000 feet, and, from the rapidity with which the corpuscular increase occurred, I imagine that acclimatisation might be complete at an altitude of 18,000 feet. But if compensation is possible up to 18,000 feet, will it still continue if a man could ascend to the summit of Everest, an altitude of 29,000 feet? At that height the weight of the atmosphere will be reduced from 760 mm. to 250 mm. of mercury, the average available supply of oxygen will be diminished to one-third and a pressure of nine tons will be removed from the surface of the body. We may well ask if man could adapt himself to changes so great as these.

There must be some limit to the degree of adaptation, and I think that in the case of this servant, who failed to adapt himself for an exceptionally long time to an altitude of 13,000 feet, there was some indication that in his case the limit of adaptation had almost been reached. Nevertheless we should be careful in forming an opinion on a single case, since the Duke of the Abruzzi, when he reached the great height in the Karakoram of 24,600 feet, experienced no inconvenience or no distressing symptoms of mountain-sickness. His ascent was comparatively slow and he had developed an acclimatisation even to such extreme heights as these. It would seem, therefore, probable that adaptation would still continue for an additional 4,500 feet, which would mark the summit of Everest. To reach such an altitude the ascent would necessarily have to be made with extreme slowness to permit of acclimatisation to gradually develop. Whatever may be the physical difficulties of the ascent, I feel certain that the physiological embarrassment on account of the rarified air is capable of being overcome.

Perhaps a residence of some weeks at a great altitude, such as the Pamirs, or Thibet, would be the best preparation for the man who is sent to compare the highest summit on the globe.

NUMBER OF RED CORPUSCLES IN THE BLOOD OF EUROPEANS RESIDING AT HIGH ALTITUDES.

The Pamir Plateau is inhabited by two nomad tribes, the Kuchiks and the Sarikolis. Most of these people live permanently at an altitude of about 13,000 feet, and seldom descend much below that level. It has been thought that people, such as the Pamir nomads, or the inhabitants of Thibet, who permanently live at great heights, possess an increased capacity of the chest for the purpose of admitting a greater volume of the more rarified air. But this is not true. The Sarikolis differ from us in no obvious anatomical respect. Their general physique and build and the volume of their chests are in no way peculiar. Their length of life and the physiological functions of their bodies resemble those of the people of the plains. They differ from us in one particular only, *viz.*, the number of corpuscles in the blood. I consider it would be a matter of interest to determine the normal blood-count of these people and to ascertain if their blood agreed in the increased number of corpuscles with that which was seen to have developed in the blood of Europeans who had ascended to these heights.

It was easy to induce the Sarikolis to give their blood in the name of science. I used to sit outside the tent with my microscope and equipment on a box before me. The nomads used to collect around in wonder at the novel sight. With a little persuasion and a promise to show them the wonders of their own blood they were very ready to come forward and were highly amused at the operations. I examined the blood of seven Sarikolis, all adult, healthy males, with the following results:—

Individual	Corpuscles per cu. mm.
Sarikoli, No. 1	7,280,000
Sarikoli, No. 2	7,060,000
Sarikoli, No. 3	7,800,000
Sarikoli, No. 4	7,760,000
Sarikoli, No. 5	7,280,000
Sarikoli, No. 6	7,220,000
Sarikoli, No. 7	7,220,000

The mean of these seven observations shows that the blood of the Sarikoli contains 7,596,000 red corpuscles in every cubic millimetre. The blood of the Kirghiz gives a similar result. I had less opportunity of examining these people, but in the blood of some who came for medical aid from the Russian side of the Frontier. I found that the blood-count reached an average of 7,920,000.

These numbers, it will be noticed, agree closely with the average count of 7,402,000 estimated in the blood of an European who had ascended to these altitudes. Thus we may conclude that the people who dwell at great heights differ from the inhabitants of sea-level by the possession of a greater abundance in the corpuscles of the blood by virtue of which they are habituated to the low pressures in which they live. Further, that the inhabitant of sea-level, during an ascent to a higher altitude, is able to improve gradually the standard of his blood until it ultimately reaches the degree of richness normal to the permanent inhabitants of the higher altitude. And when that increased development of new corpuscles has taken place the resident of the plains is as well adapted to the rarified air as is the resident of the heights. All the disturbing symptoms due to altitude will have disappeared. Acclimatisation will be complete.

Experienced observers have sometimes stated that the dwellers of great heights suffer from a distress in the same degree as those who have made a temporary ascent. But there is much likelihood of error in this respect. They have compared the people with themselves after they have made a gradual ascent, and they have observed how both suffer an equal degree of fatigue after some strenuous exertion. It is not always remembered that the man who makes the ascent has been undergoing a gradual and continuous acclimatisation until, by the time he meets with the dwellers of the higher altitudes, his physiological condition closely resembles theirs. It is very different before acclimatisation occurs. Could a man be transplanted in a single day from the plains of India to the plateau of the Pamir he would find himself involved in a bodily incapacity from which the inhabitants of that plateau would be altogether free.

#### PULSE AND RESPIRATION AT HIGH ALTITUDES.

Before passing to the reversion of the changes that occur during the descent, I will mention a few other observations on this subject that I made on the plateau. It is often thought that a marked increase in the heart's action follows on the ascent to a high altitude. But this is true

only as a consequence of exertion. After a rest there is little obvious change. Thus the average of a number of observations made at over 13,000 feet gave the pulse rate as 85, an increase which is almost negligible.

But it is different after moderate exercise. Then the pulse beats at a faster rate than is usual after an equal effort made at sea level. I once tested this at a later date by ascending about 600 feet each day for four successive days, then running a hundred yards and immediately afterwards calculating the pulse. The table indicates a gradual increase each day due to the additional 600 feet added to the height.

Date.	Altitude.	Pulse rate.
May 8th ..	5,300 feet	110
May 9th ..	5,900 "	126
May 10th ..	6,500 "	128
May 11th ..	7,200 "	140

It is very similar in the case of the respiratory mechanism. There is little change in the rate of breathing while the body is at rest, but after exertion the breathing becomes much more laboured than is usual for the same effort at sea level. Thus, in my own case, the mean of a number of calculations made at a height of over 13,000 feet while in a state of rest gave a respiratory rate of 15, a number below the normal. The great increase that follows on exertion will be evident from a table similar to that of the pulse where 600 feet were climbed each day for four successive days and the respirations counted after a run of 100 yards.

Date.	Altitude.	Respiratory rate.
May 8th ..	5,300 feet	30
May 9th ..	5,900 "	48
May 10th ..	6,500 "	51
May 11th ..	7,200 "	60

There is no increase in the rate of breathing unless undue exertion is made. Other observers have shown by careful measurement of the air that neither is there any increase in the depth of the respiration nor in the volume of air breathed. I have mentioned these facts to show that compensation for altitude does not take place through the assistance of either the heart or lungs.

## CHEYNE-STOKES RESPIRATION.

But there is one striking change in the respiration when living at great heights, a change which has also been observed in the Alps by the physiologist, Angelo Mosso. I refer to the peculiar arrest in the rhythm of the breathing known as Cheyne-Stokes respiration.

I first noticed this respiratory arrest in an European while he was asleep at an altitude of 18,000 feet. It occurred during the night and was very distinct. A long pause of nine or ten seconds would occur in the respiratory rhythm during which the breathing would completely cease. Then a gentle inspiration became audible, succeeded by two deep and laborious heavings of the chest. Then followed a fourth respiration as feeble as the first and then another pause of nine seconds during which not a breath was taken. The four respirations occupied a period of six seconds, while the silent period was as long as nine seconds.

Breathing of this type has been attributed to a diminution in the amount of carbonic acid in the blood. Carbonic acid has the power of stimulating the respiratory centre. The amount of carbonic acid in the blood diminishes with the altitude, and thus the stimulus which normally excites respiration becomes less the greater the ascent until a height is at length reached when the effects become obvious in that the breathing will temporarily cease. This periodic pause in the breathing becomes more marked the higher the altitude. I have heard it very clearly during the night at an altitude of 13,500 feet, but never so distinct as at 18,000 feet where I once counted a pause of fifteen seconds when not the slightest attempt at respiration occurred.

## RELATION OF MOUNTAIN-SICKNESS TO ATMOSPHERIC CONDITIONS.

Another factor which I noticed while on the Pamir was the relationship that exists between the symptoms of mountain-sickness and the atmospheric conditions that prevail. On two occasions we climbed a certain peak to a height of 18,203 feet. During one ascent the sky was clear and the air free from moisture, and, though we suffered a little from headache and oppression in the chest, yet the disability was slight. On the second occasion the atmospheric conditions were very different. The heavens were dark and threatening, stormy weather was imminent, and the air was saturated with moisture. Our distress on this occasion was acute. Every few paces found us panting and gasping for breath which demanded short halts at intervals of three or four minutes. On the summit we were snow-bound for two days. We lost all appetite.

were nauseated and felt not the slightest inclination for work. Even the animals were overcome, for one of our dogs vomited at this height.

It seems that there is some connection between the intensity of the symptoms and the amount of moisture in the atmosphere. Other travellers have noticed an increase in their embarrassment during the onset of a storm, others have complained that they suffer more when over rock than over snow, others more when in the sun than under cloud, others more in stagnant air than in a strong wind, and some have thought that certain mountains possess a special harmful effect.

DECREASE IN THE NUMBER OF RED CORPUSCLES DURING THE  
DESCENT FROM A HIGH TO A LOW ALTITUDE.

I come now to the descent. I have shown that the number of red corpuscles increases during an ascent from a low to a high altitude, and I will now consider the reversal of the process, *viz.* the reduction in the number of corpuscles that occurs during the return to sea-level. The production of new corpuscles during the ascent is the result of a continuous stimulus which the rarified air is always exerting on the blood-forming mechanism of the body. During the descent the person is all the time passing into a denser atmosphere and consequently this stimulus is removed. The number of corpuscles in the blood diminishes as a consequence of disuse.

The reduction in the corpuscles during the descent much resembles their increase during the ascent as is clear from this table.

Date.	Altitude.	Corpuscles per cu. mm.
August 19th	13,500 feet	7,402,000
August 20th	9,450 "	7,300,000
August 23rd	8,500 "	6,960,000
August 28th	8,000 "	6,120,000
September 6th	4,390 "	5,680,000
October 20th	500 "	5,820,000

A week after returning to sea-level the red blood remained the content normal to the altitude. But the diminution, as I think, less rapid than the increase, since ten days after our return to the plains the blood had not resumed its normal for that altitude.

REDUCTION IN THE CORPUSCLES OF A PERMANENT DWELLER AT  
HIGH ALTITUDES DURING A DESCENT.

It is clear that the dweller of a low altitude loses again the corpuscles he had manufactured as soon as he commences to return to his original level. But it is not necessarily the case that the permanent inhabitant of a high altitude will show a similar decrease in his blood if he makes the descent. The number of his corpuscles has always been high and when he descends he will meet with no stimulus to make them diminish.

I had an opportunity of investigating the question. A Sarikoli nomad accompanied our expedition on its return journey as far as Gilgit, and I made frequent examinations of his blood at different stages in the descent. This man had spent almost his whole life at an altitude of about 13,000 feet and had only on one occasion descended as low as 10,000 feet. The average blood-count of the Sarikolis has been stated to be 7,595,000 and the blood of this man agreed closely with that average. I expect that the number of corpuscles in his blood had never been much less, yet during the descent to a lower level his blood-count gradually decreased. At 9,150 feet it had diminished to 7,320,000, and at 7,500 feet it had sunk as low as 5,960,000. This was a marked diminution and its rapidity of decline corresponded closely with the blood of an European who had made only a temporary ascent to an altitude where the Sarikoli had passed his whole existence.

## CONCLUSIONS.

I will briefly recapitulate the main conclusions. Mountain-sickness is a reality which affects different persons in different ways. There is some altitude in every continuous ascent when a feeling of embarrassment will occur and the person will recognise that he has not the same capacity for effort that he possessed at sea-level. This altitude may vary for different people, in different places, and at different times, and may depend in some degree on the amount of moisture in the air. A person who ascends from a low altitude to a high altitude is able gradually to acclimatise himself to the requirements of the greater height. His method of acclimatisation is by the development of an increased number of red blood corpuscles in order to provide the body with additional oxygen-carriers to deal with the rarified air. The number of corpuscles increases in proportion to the altitude, and when a sufficient number has been developed in accordance with the altitude then acclimatisation is complete. If the ascent is gradual the manufacture of corpuscles keeps

pace with the requirements of the altitude and no inconvenience is felt. If, on the other hand, the ascent is rapid, then a sufficient number of corpuscles has not time to develop: the blood is relatively deficient and the person suffers from mountain-sickness. If he remains for an adequate time at the altitude he has reached, he will gradually become acclimatised and his distress will disappear. In certain persons the development of corpuscles is delayed and their sufferings as a consequence are acute and prolonged. People who live permanently at a high altitude have a richer blood supply than those who inhabit low altitudes but differ in no other respect. The number of corpuscles in the blood of a dweller at low altitudes can increase until it reaches ultimately the number normal to the dwellers at high altitudes. And during a return to sea-level the number of corpuscles will again diminish until the normal for the altitude is again restored, and the blood of a permanent inhabitant of high altitudes will undergo a similar change. The human body has a wonderful power of adjusting itself to changes in its environment. It can easily adapt itself to an atmosphere that contains only half the oxygen supply available at sea-level, and there is reason to believe that it would adapt itself to one-third of this supply such as it would find on the summit of Everest.

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THE CORRELATION BETWEEN THE CHEMICAL  
COMPOSITION OF ANTHELMINTICS AND  
THEIR THERAPEUTIC VALUES IN  
CONNECTION WITH THE  
HOOKWORM INQUIRY  
IN THE MADRAS  
PRESIDENCY.

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[Received for publication, February 1, 1921.]

XI. EXTRACTUM FILICIS LIQUIDUM.

HISTORY.

The rhizome of male fern has been a favourite anthelmintic since the times of Theophrastus, Dioscorides, Pliny and Galen. It seems to have been generally administered in 36 grains doses, either alone or mixed with some other substance or substances.

Valmont de Bomare (1764)<sup>1</sup> says that the root of male fern was very much used by the ancients in chronic diseases as an aperient and antispasmodic. He adds that it is also a powerful vermifuge, and one of the greatest secret remedies of Empirics who cleverly administer it along

with some mercurial preparation, to expel round-worms and flat-worms from the body. But pregnant women ought not to be treated with this drug as it may cause abortion.

About the year 1775, the King of France purchased from Madame Nuffer, the widow of a Swiss surgeon, a secret remedy for tapeworm, which proved to be the powdered root of male fern.

Marinus (1824) administered male fern along with sulphuric ether. In 1825, Peschier fought the *Bothriocephalus* infection then prevailing in Geneva with an ethereal extract prepared by his brother. Buchner, in 1826, either used an alcoholic extract or the oleoresin.<sup>22</sup>

In 1879, Perroncito tried the direct action of several drugs on larvæ of *Ankylostomum* and *Strongyloides*, and found that the ethereal extract of male fern, when pure, killed them in less than ten minutes. He accordingly began to treat cases of ankylostomiasis with the extract, and the results proved satisfactory.<sup>2, 3, 6</sup> These were soon confirmed by Parona<sup>5</sup> who reported the expulsion of 1,250 ankylostomes and permanent disappearance of ova from the feces.

The male fern treatment followed the ankylostome infection in its spread over Continental Europe, and its efficiency was repeatedly asserted. Out of 21,612 cases treated in Westphalia by Bruns the drug failed to expel all the parasites in only 1·5 per cent, and in only 15 to 30 per cent of cases was it necessary to give a second dose. The method adopted by the doctors of the Knappschaftverein in Westphalia may help to understand the nature of the treatment long in vogue in most of the coal mines in Europe<sup>11</sup> :—

*Monday*—evening—0·25 gramme jalap—0·25 gramme calomel.

*Tuesday*—morning—8 grammes Extract Male Fern with 20-30 grammes senna.

evening—light meal.

*Wednesday*—morning—rest.

evening—0·25 gramme jalap—0·25 gramme calomel.

*Thursday*—morning—8 grammes Extract Male Fern with 20-30 grammes senna.

evening—meal.

*Friday*—morning—rest.

evening—0·25 gramme jalap—0·25 gramme calomel.

*Saturday*—morning—4 grammes Extract Male Fern with 20 grammes senna.

evening—discharge.

In many cases the extract of male fern was associated with one or more drugs, whose presence may have enhanced the efficiency of the treatment. As early as 1889, Sonderegger<sup>4</sup> combined male fern and santonin. A little later, Hermann at Muns, Mdyeg and Lumbinet at Laege, used 4 grammes extract and 3 grammes chloroform.<sup>4</sup> This mixture together with half a drop of croton oil eventually became proprietary as "Deboncoeur's Teufuge fraigais."<sup>10</sup> Troussseau and Pidoux,<sup>9</sup> and Nathan Larrier<sup>17</sup> combined *filix mas* with ether. DeCosta (1913)<sup>18</sup> used a mixture of ethereal oil of male fern and thymol, of each 2 to 4 grammes, santonin 1 decigramme, calomel 6 to 8 decigrammes, made into six boluses to be taken fasting at intervals of ten minutes; the dose to be repeated next day; 58 per cent cures were obtained with one treatment.

The male fern treatment does not appear to have found much favour outside Europe. Sandwith (1887)<sup>8</sup> of Cairo, had nothing but disappointment. Ashford and King (1907)<sup>12</sup> say the ethereal extract gave good results in some cases and in others it did not. Schüffner (1912)<sup>16</sup> treated 21 cases with 10-12 grammes of extract and only 7 worms were removed out of a total of 1,260 hookworms.<sup>20</sup> Greisert (1913)<sup>19</sup> held opinion that male fern is unreliable for use in the tropics and has no conspicuous superiority over other anthelmintics. Patterson (1908),<sup>13</sup> Mollet and Carrero (1916)<sup>21</sup> found the drug to be inefficient in the treatment of hookworm cases.\*

The ethereal extract of male fern is known to have caused severe poisoning in man. Poulsson<sup>7</sup> has collected up to the year 1894, 46 cases of poisoning by male fern. Schultz<sup>15</sup> reports that Sidler Huguenin (1898) compiled 78 cases of poisoning and 12 deaths. Nielsen 19 cases. Most of the cases of poisoning recorded have been the result of exceeding the admissible dose, 8 grammes, or repeating the unsuccessful treatment on the following day.

#### COMPOSITION AND PROPERTIES.

Extract of male fern is a thick, dark green oily liquid obtained by exhausting with ether the powdered rhizome of *Asplenium Filix Mas*, Swartz.

The composition of the drug has been the subject of considerable study and many are the substances which have been isolated, but as

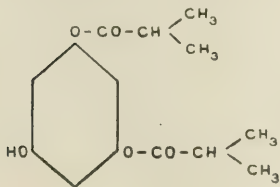
\* The toxicological properties of Male Fern do not appear to have been thoroughly investigated although the plant is most common on the hills. Toxic 4,000 to 10,000 mg. when dried 23.

spite of all the work done, there is so far no consensus of opinion as regards either the chemical nature of the different constituents or their respective therapeutic values. This is not to be wondered at if we bear in mind that the active principles of male fern readily deteriorate not only when isolated as such, but also when combined as in the crude extract and even in the dried rhizome itself.

For the purpose of this investigation only two of the active principles of male fern—*filicin* and *filmaron*—need be taken into consideration.

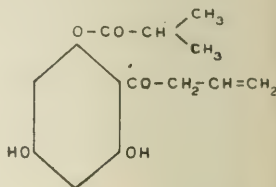
Filmaron is a brownish yellow powder melting at  $60^{\circ}$ , insoluble in water, difficultly soluble in cold alcohol, but very soluble in other general solvents. This compound, considered by many to be the true anthelmintic constituent of male fern, forms about 5 per cent of the extract. Its formula is  $C_{47} H_{54} O_{16}$ .

The other different constituents seem to be largely decomposition products of filmaron. By far the most important of these is filicin or filicic acid, which constitutes the major portion of the sediment the extract deposits on standing. It occurs both in an inactive crystalline form and an amorphous active form with molecular formula  $C_{14} H_{16} O_5$ . Filicin was at first supposed to be an isobutyric derivative of hydroxynaphthoquinone; but, as under the influence of saponifying agents, it breaks up into isobutyric acid and phloroglucinol, it is now considered to be a phloroglucine derivative.



PHLOROGLUCINEDIIISOBUTYRATE

I



ISOBUTYROPHLOROGLUCYLALLYLKETONE

II

Experience has shown the extract of male fern to be a tæniacide, and experiments have proved its active principles to be both neurotoxic and myotoxic poisons.

In man, the first symptoms of poisoning are due to gastro-intestinal irritation, which causes nausea, vomiting and purging; later stupor and

tautness or even convulsions may develop while cardiac weakness, disturbances of respiration, yellow vision, amblyopia and sometimes permanent blindness due to optic atrophy, may also result. Death is usually preceded by delirium and muscle cramps.

It is generally admitted that the constituents of the drug are absorbed with extreme difficulty; but, it must be remembered that there is no characteristic reaction for male fern.

#### ANTHELMINTIC VALUE.

The three samples of extract we used were obtained from different sources and at different times. They were all found to be unadulterated and their physical properties agreed with those given by the *Pharmacopœia*. The barium hydroxide method showed the presence of 19, 24, 25 per cent. "Crude filicin," respectively.

Our cases were all healthy adult male convicts. The extract was thoroughly shaken with gum acacia emulsion and given in two portions at an hour's interval on an empty stomach. Epsom salts both preceded and followed the administration of the drug. No food was allowed until the bowels had moved.

The total hookworm content was determined in our usual routine way, 60 grains thymol being used as a subsequent treatment.

We adopted a 90 minims dosage, corresponding to the 6 grammes recommended by American authors, and nearly all the cases bore the treatment well.

TABLE.

*Number of hookworms removed by one test treatment of Ext. Filicis Liq.*

TEST TREATMENT.				Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.		
Extract Number.	Specific gravity.	Filicis per cent.	Dose.			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
I	0.990	19%	90 minims	8	Test treatment ..	0	1	1	0.0	0.1	0.1
					Subsequent treatment ..	10	604	614			
					Total hookworms ..	10	605	615			
II	1.034	24%	Ditto	28	Test treatment ..	2	28	30	10.0	5.3	5.5
					Subsequent treatments ..	18	492	510			
					Total hookworms ..	20	520	540			
III	1.000	25%	Ditto	15	Test treatment ..	0	7	7	0.0	5.7	5.4
					Subsequent treatments ..	9	114	123			
					Total hookworms ..	9	121	130			

The following results were recorded :—

1. In a series of 51 cases treated, 38 hookworms were removed out of a total of 1,285. The worms expelled were dead, but retained their natural shape.

2. No ascarids were removed though round-worms were present in 14 cases and whipworms in eight.

3. No tapeworms were passed though 6 cases were infected with them.

#### CONCLUSIONS.

1. Extract of male fern is a mixture in which the several constituents are present in varying proportions. It deteriorates readily and may easily be adulterated.

2. Very little is known of the chemistry and pharmacology of the drug.

3. 90 minims is the admissible dose.

4. Extract of male fern cannot be recommended as an anthelmintic.

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[Received for publication, February 1, 1921.]

XII. KOUSSO.

HISTORY.

Koussou, the dried female flowers of *Brayera anthelmintica*, Kunth (=*Hagenia abyssinica*, Gmel.), has long been used as a taniacide, and although dropped from the United States Pharmacopœia, it has been introduced into the National Formulary and is retained in the British Pharmacopœia.

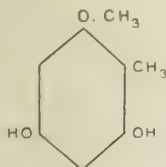
In 1905 Calmette and Breton<sup>3</sup> reported that Koussou had been tried in ankylostomiasis, but had proved inefficient. This is, as far as we know, the only mention of the drug with reference to the treatment of hookworm infection.

## COMPOSITION AND PROPERTIES

Various compounds have been obtained from extracts of Kousso flowers. It will be sufficient to mention here *Kosin* and *Kosotoxin* which have been both credited with the physiological properties of the drug.

Kosotoxin is an amorphous pale yellow powder, melting at  $80^\circ$ , insoluble in water, but soluble in the other general solvents. Its formula is  $C_{28}H_{34}O_{10}$ . When boiled with barium hydroxide it yields kosin. It would thus appear that kosotoxin is the parent substance of the various constituents which have been isolated from the original drug.

Commercial Kosin essentially consists of yellow rhombic crystals, which melt at  $112^\circ$ . The crystals are insoluble in water but easily soluble in alcohol, ether, benzene, glacial acetic acid, and in alkalis. By repeated crystallization from alcohol Boelm and Lobeck<sup>2</sup> have obtained from kosin two compounds, *alphakosin*— $(C_{22}H_{26}O_7)$ —melting at  $160^\circ$ , and *betakosin*— $(C_{21}H_{26}O_7)$ —more soluble in alcohol and melting at  $120^\circ$ . When treated with zinc dust and aqueous sodium hydroxide kosin splits into isobutyric acid and the monomethyl ether of methylphloroglucinol.



METHYLOXYMETHYLPHLOROGLUCINOL

Kousso, then, contains substances which, like the active constituents of the various ferns, are compounds of isobutyric acid with members of the phloroglucinol series. And it has been shown that in the lower animals, kosotoxin is a powerful muscle poison, resembling fibroin.

The toxic symptoms in man rarely go beyond nausea and vomiting. The drug is, however, considered a dangerous one in Abyssinia, and is said to be especially so in the case of pregnant women, causing abortion and killing both mother and child.<sup>1</sup>

## ANTHELMINTIC VALUE

Through a regrettable oversight the whole amount of Kousso of our possession was used before the percentage of ash had been determined.

An infusion was prepared by gradually mixing the powder with 8 ounces of warm water and allowing the mixture to stand for fifteen minutes. It was then stirred and administered in three draughts at intervals of half an hour. A purgative was only given to those cases whose bowels had not moved within four hours from the time of the administration of the last dose. The dosages varied from 2 to  $5\frac{1}{2}$  drachms. No toxic symptoms and no after affects were noticed.

TABLE .

*Number of hookworms removed by one test treatment of Koussou.*

Experiment number.	TEST TREATMENT.	Number of cases treated.	HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.		
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
1	$3\frac{1}{2}$ drachms ..	1 { Test treatment	14	19	33	100.0	76.2	86.9
		1 { Subsequent treatments	0	5	5			
		Total hookworms ..	14	24	38			
2	2-4 drachms ..	6 { Test treatment	0	0	0	0.0	0.0	0.0
		6 { Subsequent treatments	0	21	21			
		Total hookworms ..	0	21	21			
3	$4\frac{1}{2}$ - $5\frac{1}{2}$ drachms ..	6 { Test treatment	0	2	2	0.0	5.5	3.0
		6 { Subsequent treatment	29	34	63			
		Total hookworms ..	29	36	65			

The following results were recorded :—

1. One case who had received  $3\frac{1}{2}$  drachms of the drug passed 33 hookworms out of a total of 38. The removal went on for three consecutive days, on which 16, 11, and 6 worms were expelled, respectively.

2. In a series of 12 cases treated with doses varying from 2 to  $5\frac{1}{2}$  drachms, only 2 hookworms were removed out of a total of 86.

3. Neither roundworms nor whip-worms were removed though 7 cases were found infected with the former and 2 with the latter.

Kousso cannot, therefore, be recommended as an anthelmintic.

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XIII. KAMALA.

HISTORY.

KAMALA consists of the minute glands and hairs which cover the capsules of *Mallotus philippinensis*, Muell. Arg. (= *Rottleria tinctoria* Roxb.), a small, evergreen tree, found throughout Tropical India.

In most European and American works it is stated that Kamala has long been used in India in the treatment of tapeworm; but neither literature nor tradition bear this out, and we are led to admit that it was not until well into the nineteenth century that the value of the drug as an anthelmintic was recognised. That this was overrated may be gathered from the different opinions held by medical men, as well as from

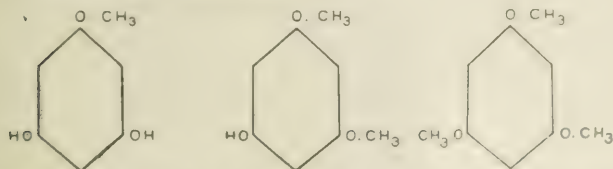
the fact that the drug was soon discarded from both the British and United States Pharmacopœias which had recognised it as official.

Speaking of kamala, Waring (1874)<sup>1</sup> says :— "In medicine, the purplish red powder has attained considerable repute as a remedy for *Taenia* or tape-worm. It has little or no effect on other form of intestinal worms." In 1912, Tarapore<sup>3</sup> found that an old sample of the drug caused the removal of thread-worms, round worms, and hookworms. He accordingly tried a fresh stock which, however, proved useless.

#### COMPOSITION AND PROPERTIES.

Kamala occurs as a granular brick-red powder, odourless and nearly tasteless. It is insoluble in cold water and but very slightly soluble in boiling water; but alkaline solutions, alcohol, and ether dissolve a large proportion of it forming a deep red solution. Commercial varieties are often highly adulterated with sand, earthy impurities, red brick dust, etc. The amount of impurity is usually estimated by heating to redness and weighing the residue. A good specimen ought not to contain more than 8 per cent of mineral matter.

Numerous chemists have investigated kamala, and not less than six distinct substances have been isolated. The most important of these is *rottlerin*,  $C_{33}H_{20}O_9$ , which forms salmon coloured plates readily soluble in ether. When treated with barium hydroxide solution or with zinc powder and sodium hydroxide, rottlerin gives the mono-, di-, and tri-methyl ethers of phloroglucinol.



Rottlerin is, therefore, a derivative of phloroglucinol and somewhat related to flicin of male fern and kosin of kousso.

Semjei<sup>2</sup> has shown that kamala is toxic to frogs, tadpoles, and worms. The symptoms produced in frogs are similar to those resulting from the action of substances obtained from different ferns, the paralytic effect on motor nerves and muscles being very striking. The cathartic action of kamala is, however, peculiar. In full doses it is not only a powerful, or even violently, purgative, causing in many cases considerable haemorrhage

and griping. Whether the pharmacological properties of kamala reside in rottlerin alone or in the mixture has not yet been ascertained.

According to Semper, who experimented on dogs, kamala and its products are not absorbed, another property which Male Fern has been credited with.

#### ANTHELMINTIC VALUE.

The sample we used contained 34 per cent of impurity.

The drug was administered in dosages ranging from 1 to  $2\frac{1}{2}$  drachms given in one portion without any purge. The powder was swallowed down with the help of water. All our cases bore the treatment well, but the  $2\frac{1}{2}$  drachms dosage caused violent purging and was not repeated. No after effects were noticed.

TABLE.

*Number of hookworms removed by one test treatment of Kamala.*

Experiment number.	Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.				PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.			
				A. duodenale.	N. americanus.	A. duodenale and N. americanus.		A. duodenale.	N. americanus.	A. duodenale and N. americanus.	
1	1=2 drachms ..	5	Test treatment ..	0	1	1		0.0	0.3	0.3	
			Subsequent treatment:	29	366	395					
			Total hookworms ..	29	367	396					
2	2= $2\frac{1}{2}$ drachms ..	2	Test treatment ..	0	0	0		0.0	0.0	0.0	
			Subsequent treatment:	0	11	11					
			Total hookworms ..	0	11	11					

The results showed that :—

1. In a series of 7 cases only one hookworm was removed out of a total of 407.

2. No ascarids were removed though round-worms were present in 3 cases and whip-worms in 5.

Kamala cannot, therefore, be recommended as an anthelmintic.

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XIV. PULVIS ARECÆ.

HISTORY.

THE areca or betel-nut palm—*Areca Catechu*, Linn—is indigenous to the Sunda Islands, but is now widely cultivated in tropical countries of the Far East, where the seeds mixed with lime and betel pepper—*Piper Betle*, Linn—form the masticatory so well known by the name of *Betel*.

In China and India the ground areca nut is used as an anthelmintic, and its supposed efficacy in promoting the expulsion of both the tapeworm and the round-worm in the human subject has led to its introduction into the British Pharmacopœia.

Barelay, who appears to have been the first practitioner who called attention to the remedial value of the areca nut, the betel nut of commerce, in the expulsion of tapeworm, administered it, in powder, in doses of from four to six drachms, stirred up with milk.<sup>1</sup> Powell<sup>6</sup>

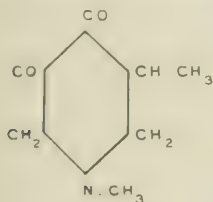
found betel nut and leaves' juice an efficient anthelmintic to the extent of an ounce or more.

At the British Medical Congress held in 1900, Mason quoted Powell as saying that betel nut chewing is possibly a protective habit in the natives of Assam, etc., acquired instinctively in consequence of its prophylactic virtue against the ankylostomiasis.<sup>7</sup> However, some thirty years before, Waring had remarked: 'Anthelmintic virtues have been assigned to the nut, but it can hardly have any claim to this character, as amongst the Hindus and Burmese, who use it habitually as a masticatory, intestinal worms are almost universally met with.'<sup>4</sup>

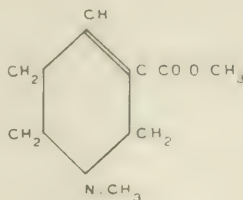
Bentley (1904)<sup>8</sup> treated 6 hookworm infected cases with *pulvis areca*, administered in two half ounce doses, and found the drug inferior to both thymol and betanaphthol; but no results are recorded. Schüffner (1912)<sup>9</sup> reports having treated twenty cases with *Areca Catechu* in 30-40 grammes doses; only 8 worms were removed out of a total content of 751 hookworms.

#### COMPOSITION AND PROPERTIES

The seeds were first examined by Bombelon (1886)<sup>2</sup> who isolated a volatile alkaloid resembling nicotine which he called *arechina*. Later Jahns<sup>3, 5</sup> found four different alkaloids in areca nut: *arecaine*, to the extent of about 0.1 per cent; *arecoline*, 0.07-0.1 per cent; *arecolidine*, and *guvacine*, in smaller quantities.



ARECAINE



ARECOLINE

According to Jahns *arecaine* is the active principle of the areca nut and a powerful tæniacide. Other authors believe *arecaine* physiologically inactive and consider the medicinal properties of the nut are due to *arecoline*. *Arecoline hydrobromide* is recognised in several pharmacopœias of Continental Europe. It is a highly toxic substance used as a analogue and diaphoretic, and said to be anthelmintic.

Areca nut is also considered to be a powerful astringent which, by its internal use, tends to counteract the relaxation of bowels to which the heat of a tropical climate so strongly predisposes.<sup>10</sup>

#### ANTHELMINTIC VALUE.

The nuts were cut into small bits and dried in the open, but in the shade. On grinding we obtained a brown coloured powder which we administered in one single portion, early in the morning, without any previous preparation of the patient. It was found that a 6 drachms dosage caused griping and pain, and four liquid motions. The dosage was, therefore, limited to 4 drachms which produced no symptoms and were followed by one to three free semi-solid stools.

As no worms were passed with the powdered dry nut, we treated four cases with 4 drachms of powdered fresh nut. Irritation of the intestinal tract was marked by three to seven very watery stools, the effect of the treatment being still noticeable the day after. However, no after effects were noticed.

TABLE

*Number of hookworms removed by one test treatment of Pulvis Arecæ.*

Experiment number.	Treatment.	Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.		
				A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
1	Dry areca nut powder 4 drachms.	4	Test treatment ..	0	0	0	0-0	0-0	0-0
			Subsequent treatments	9	124	133			
			Total hookworms ..	9	124	133			
2	Do. 6 drachms..	2	Test treatment ..	0	0	0	0-0	0-0	0-0
			Subsequent treatments	0	9	9			
			Total hookworms ..	0	9	9			
3	Fresh areca nut powder, 4 drachms.	4	Test treatment ..	0	0	0	0-0	0-0	0-0
			Subsequent treatments	0	99	99			
			Total hookworms ..	0	99	99			

Our notes show that :

1. In a series of 10 cases *no* hookworm was removed though subsequent treatments showed the presence of 232 necators and 9 ankylostomes.

2. Two cases were found infected with round-worms and two with whip-worms, but *no* worm was expelled by the areca nut treatment.

*Pulex arceut* cannot, therefore, be recommended as an anthelmintic.

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# A BACTERIOLOGICAL INVESTIGATION OF INFLUENZA.

## Part II.

### CARRIED OUT UNDER THE INDIAN RESEARCH FUND ASSOCIATION.

BY

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[Received for publication, February 1, 1921.]

*Influenza in Bombay, July 1919 to June 1920.*

WHEN the investigation commenced in Bombay the city had already, in 1918, passed through two epidemics of influenza. The third commenced in May 1919, reached its height in June and July and then gradually declined, so that by the end of October it had practically come to an end as far as Bombay city was concerned.

From the outset the disease was of a milder type than in the previous epidemics and milder too than in the Calcutta epidemic earlier in the same year.

The cases examined in Bombay were those diagnosed as influenza, bronchitis, broncho-pneumonia and lobar pneumonia at the J. J. Hospital and at the Labour Corps War Hospital, Dadar. In the former hospital the patients usually arrived during the second week of the disease, whilst, in the latter, I was able to observe many cases from the very onset of the attack. In this hospital the patients were all men of the Labour Corps coming from Mesopotamia and Burma as well as from various parts of India. There is no doubt that the majority of the Mesopotamia cases had developed the disease before arriving in Bombay (*i.e.*, either in Basrah, or on board a transport), and the same may be said of batches of men admitted to the Labour Corps Depôt from Jubbulpore.

The clinical picture presented by the disease among the patients at both those hospitals was similar to that noted in Calcutta and described elsewhere.(1)

It was remarked, however, that diarrhoea and meningeal symptoms were very rare. The respiratory affection gave rise in general to the same signs and symptoms as were observed in Calcutta, but the prostration and toxic appearance of the patients were less marked.

Meningeal symptoms, apart from drowsiness and apathy, were so infrequent that on one occasion only was I asked to examine a specimen of cerebro-spinal fluid and this was proved to contain the meningococcus. The mortality here was comparatively low (about 10 per cent) only one quarter of what it was in the Campbell Hospital, Calcutta, during the epidemic of the early part of 1919.

While the presence of an epidemic of influenza is readily recognised, the diagnosis of individual cases of influenza is often a matter of doubt. At the beginning and to a less extent at the end of the epidemic cases were diagnosed acute bronchitis, broncho-pneumonia or lobar pneumonia which, during the height of the epidemic, would have been labelled influenza.

The physician in charge of one ward might diagnose certain cases as acute bronchitis or broncho-pneumonia, while the physician in another ward would consider other similar cases to be influenza, the difference being largely due to the individual's conception of influenza. As the epidemic declined itself a change in mental attitude would result in all such cases being recorded as influenza.

In one institution (in Calcutta) the reason for a large increase of admissions to hospital for supposed dengue fever only became obvious when, in the course of another week, the already existing influenza became manifest in epidemic form.

The attitude adopted here, as in the previous report, has been to consider all cases diagnosed as influenza, bronchitis, broncho-pneumonia and lobar pneumonia (not due to pneumococcus) occurring during the epidemic as influenza.

#### BACTERIOLOGICAL EXAMINATION OF SPECIMENS

The examination of sputum and pharyngeal smears was carried out on the lines previously adopted.

One per cent heated pigeon blood agar and 2 per cent unheated rabbit or human blood agar (PH 7.2 to 7.4) were used for isolating the organisms and each specimen planted on both media.

During the monsoon months in Bombay 2000 colonies were experienced in keeping Petri dishes containing blood agar for 24 hours.

more than a day or two at a time, and so the ordinary plating method was discarded in favour of the platinum loop method described by Liston and Gorié for the examination of fæces, in which agar slopes are used instead of Petri dishes.(2)

The blood agar slopes could be prepared in large numbers and stored in the ice box for several weeks with little fear of contamination, and with proper trituration and dilution of the samples of sputum discrete colonies could be obtained as readily as on plates.

The method of 'plating' sputa was as follows:—

A suitable fragment of sputum is taken up in a platinum loop of about 5 millimetres diameter, thoroughly trituated in 1 c.c. of saline, and from this suspension dilutions approximately 10-fold, 100-fold and 1000-fold are made by means of the loop. One loopful of each of the three dilutions is smeared over the surface of a *perfectly dry* blood-agar slope. Usually the second tube of the series will give discrete colonies; with sputa very poor and very rich in bacterial content the first and the third tubes, respectively, will give the desired result.

The results of the examination of sputum and pharyngeal secretion are shown in Tables I to V.

TABLE I.

*Results of the examination of samples of sputum from influenza patients collected during the year July 1, 1919 to June 30, 1920.*

		No. of specimens examined.	No. of times each organism was isolated.
Isolated on 1% heated pigeon blood agar.	Pfeiffer's bacillus .. ..	243	166-68%
	Catarrhalis group .. ..	243	102-42%
	Diphtheroids .. ..	243	95-39%
	Staphylococcus aureus .. ..	243	67-28%
Isolated on 2% unheated rabbit or human blood agar.	Streptococcus (non-hæmolytic) .. ..	220	197-90%
	Pneumococcus .. ..	220	131-60%
	Streptococcus (hæmolytic) .. ..	220	19-9%

*Notes.*—(1) 220 specimens were planted on 1 per cent heated pigeon blood agar as well as upon 2 per cent unheated rabbit blood agar, while 23 specimens were planted on the former medium only. The heated blood medium is not suitable for pneumococcus and streptococcus on account of the impossibility of detecting hæmolysis and pigmentation, but is a splendid medium for Pfeiffer's bacillus.

(2) In this, as in the other tables, it is to be noted that only one specimen was taken from each patient or healthy person.

TABLE II.

Showing the presence of Pfeiffer's bacillus, pneumococcus and streptococcus in the sputum of influenza patients at different periods during and after the epidemic.

	Date	PFEIFFER'S BACILLUS.		STREPTOCOCCUS NON-HEMOLYTIC.		PNEUMOCOCCUS.	
		No. of specimens examined	No. of times isolated.	No. of specimens examined	No. of times isolated.	No. of specimens examined	No. of times isolated.
During the epidemic	July 1919 ..	44	33-75%	40	36-00%	40	22-55%
	August " ..	42	32-76%	40	38-05%	40	20-50%
	September " ..	38	28-74%	36	34-44%	36	19-55%
	October " ..	29	20-69%	25	21-84%	25	15-60%
	TOTAL ..	153	113-74%	141	129-91%	141	78-20%
After the epidemic	November 1919	16	10-63%	14	12-86%	14	10-71%
	December " ..	18	11-61%	16	14-88%	16	12-75%
	January 1920 ..	14	8-60%	12	10-83%	12	9-77%
	February " ..	12	7-58%	11	10-91%	11	8-73%
	March " ..	12	7-58%	10	8-80%	10	7-70%
	April " ..	8	4-50%	7	6-80%	7	4-47%
	May " ..	6	4-66%	5	4-80%	5	3-33%
	June " ..	4	2-50%	4	4-100%	4	2-50%
	TOTAL ..	90	53-59%	79	68-80%	79	55-70%

TABLE III.

Showing the presence of Pfeiffer's bacillus, pneumococcus and streptococcus (non-hemolytic) in the throats of healthy persons during and after the epidemic.

Period during which the swabs were taken.	Where obtained.	No. of swabs examined.	No. of times isolated.		
			Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.
During the epidemic (August 1919)	Labour Corps	83	14-17%	39-47%	
	Depot-Dachau	78	" "	21-27%	61-78%
After the epidemic (April 1920)	Bombay Bio-technological Laboratory	50	11-20%	28-56%	
		50	4-80%	" "	12-24%
After the epidemic (June 1920)	H. M. Common Prison, Bombay	60	5-80%	25-42%	

TABLE IV.

*A comparison of the occurrence of Pfeiffer's bacillus, pneumococcus and streptococcus in the sputum of influenza patients and the naso-pharyngeal secretion of healthy persons during and after the epidemic.*

	INFLUENZA PATIENTS.		HEALTHY PERSONS.	
	No. of cases examined.	No. of times organism was isolated.	No. of cases examined.	No. of times organism was isolated.
<b>PFEIFFER'S BACILLUS--</b>				
During the epidemic (a) ..	153	113-74%	138	25-18%
After the epidemic (b) ..	90	53-59%	188	15-8%
<b>PNEUMOCOCCUS--</b>				
During the epidemic (a) ..	141	76-54%	138	67-48%
After the epidemic (b) ..	79	55-70%	188	61-32%
<b>STREPTOCOCCUS --</b>				
<i>(non-hæmolytic):</i>				
During the epidemic (a) ..	141	129-91%	138	118-85%
After the epidemic (b) ..	79	68-86%	188	153-81%

(a) Influenza patients examined .. July to October 1919.

Healthy persons examined .. August and September 1919.

(b) Influenza patients examined .. November 1919 to June 1920.

Healthy persons examined .. April to June 1920.

Reference to Table I shows that in Bombay the predominating organisms in the sputum of influenza patients have been Pfeiffer's bacillus, pneumococcus and non-hæmolytic streptococcus.

Pfeiffer's bacillus and streptococcus have been about as prevalent in the Bombay epidemic as in the Calcutta epidemic of 1918-19. Pneumococcus, however, does not seem to have been so closely associated with influenza in the former city as in the latter, and this is borne out by the results of the bacteriological examination of the lungs and accessory respiratory sinuses post-mortem.

In Table II the results of the bacteriological examination of the sputa of influenza patients are shown month by month. Unfortunately

the number of specimens examined after November 1919 has been rather small, owing to the decline of the epidemic. It will, however, be seen that there was a distinct drop in the incidence of Pfeiffer's bacillus after the epidemic was over. The streptococcus incidence remained practically the same throughout the year and appeared to be independent of the presence of influenza. Pneumococcus showed a rise between November and March and then a period of decline.

A possible explanation of these findings is as follows :—

(1) Pfeiffer's bacillus had a direct connexion with influenza in Bombay, while the presence of non-hæmolytic streptococcus was more or less accidental.

(2) Pneumococcus, though an important secondary invader and a potent cause of death, was probably not such an important factor in the influenza of Bombay as it was in Karachi, 1918, and in Calcutta, 1919. The rise during November to March may have been a seasonal one, as this is the time of the year (the cold season) when lobar pneumonia is most prevalent in Bombay as in many other parts of India.

Tables III and IV bring out several important points :

(1) More than twice as many healthy persons harboured Pfeiffer's bacillus in their throats during the epidemic than after it had subsided.

(2) Pfeiffer's bacillus was found more than four times as frequently in the throats of influenza patients as in healthy persons, while there was no such marked difference in the case of pneumococcus and non-hæmolytic streptococcus.

(3) Non-hæmolytic streptococcus was equally present in influenza patients and in healthy persons during and after the epidemic.

(4) Pneumococcus was more prevalent in healthy persons during the epidemic than after.

These results are taken as additional evidence of the essential connexion of Pfeiffer's bacillus and the accidental connexion of non-hæmolytic streptococcus with the influenza epidemic in Bombay.

As was noted in the introductory section of this report, many of the patients at the Dader Labour Corps Hospital have discussed some the onset of their attack. Some of those were mild cases and suffered nothing beyond a transient catarrh, conjunctivitis, coryza, prostration and fever for three or four days; others developed bronchitis and bronchopneumonia. A routine examination of the sputum of pharyngeal secretion of these patients was carried out and the results placed in Table V.

TABLE V.

*Results of the examination of sputum or pharyngeal secretion of influenza patients (mild and severe cases) at the Dadar Labour Corps Hospital, July to September 1920.*

A	B
CASES OF "CATARRH" AND TRANSIENT FEVER WITHOUT BRONCHITIS OR PNEUMONIA.	CASES OF "CATARRH" FOLLOWED BY BRONCHITIS AND/OR PNEUMONIA.
No. of specimens examined, 48.	No. of specimens examined, 38.
Pfeiffer's bacillus .. 38 — 79%	26 — 68%
Pneumococcus .. 21 — 44%	27 — 71%
Streptococcus .. 41 — 85%	34 — 89%

It will be seen :—

(1) That Pfeiffer's bacillus was isolated somewhat more frequently from the mild cases than from the severe cases, although the difference is not striking. This, I believe, may be explained in the light of the results of the post-mortem examination of the lungs and accessory respiratory sinuses (q. v). Pfeiffer's bacillus tends to be out-grown by hardier organisms, and seems to seek the remoter portions of the respiratory tract. It is common enough to find this organism in pure or almost pure culture in the droplets of pus which exude from the cut ends of the fine bronchioles and in the pus of the accessory sinuses, when scrapings from the trachea or bronchi may show Pfeiffer's bacillus in small number and other organisms (*e.g.*, streptococcus, catarrhalis group, etc.) in abundance.

(2) That non-hæmolytic streptococci are equally present in mild and severe cases.

(3) That pneumococcus shows a striking predominance in the severe cases.

An interesting feature was the appearance of a pleomorphic non-hæmolytic streptococcus which was not noticed in the bacterial flora of the sputa examined until the arrival of the Basrah cases at Dadar. The colonies of this organism seemed to be identical in appearance with

those of the ordinary non-haemolytic streptococci of the respiratory tract. The organisms themselves, however, were extremely pleomorphic, growing in short chains or pairs in broth. The individual cocci varied greatly in shape and size, often diphtheroid in appearance, carrot-shaped or wider in the middle than at the ends, and those oddly shaped organisms often occurred in the same chain along with cocci of normal appearance. Six such strains were tested and found to have the following characters: (1) very pathogenic for mice; (2) insoluble in bile; (3) capable of fermenting glucose but not mannite and of acidifying and clotting milk.

Although it is improbable that this organism plays an important part in influenza, yet its presence in a series of cases from one area opens up a field for speculation.

*Discussion.*—It is remarkable that Pfeiffer's bacillus was found so frequently in some localities and in some epidemics, while at other times and in other places trained bacteriologists confessed their failure to isolate this elusive organism.

I am inclined to believe that the use of improper culture media must have been the cause of failure in many instances. In my own case it was the cause of failure when the investigation was commenced in 1919, and I have no doubt that other workers encountered difficulties similar to mine.

This organism was frequently found in Calcutta, as soon as experience had taught proper methods of isolating it. In Bombay it was more often encountered in the sputum of influenza patients and in the pharyngeal secretion of healthy persons during the height of the epidemic than at its termination.

The pleomorphic streptococcus noticed in the Basrah patients was not recognised in either Bombay or Calcutta patients but may possibly have been of common occurrence in the influenza of Mesopotamia.

A non-haemolytic streptococcus has been found in practically every case in Bombay and was apparently as common in healthy persons and influenza patients at the end of the epidemic as it was at the beginning. It is thus considered to have little connection with the influenza epidemic.

Pneumococcus was not as common in Bombay as it was in Calcutta and was more prevalent during the period November to March (the cold season) than at the height of the epidemic. The comparatively infrequent occurrence of this organism may be an explanation of the mildness of the Bombay epidemic.

From the evidence it seems not improbable that the 'secondary invaders' of the respiratory tract of persons suffering from influenza will vary with the locality in which an epidemic occurs, with the particular phase of the epidemic and with its recrudescences from time to time.

As far as the epidemics investigated in India in 1919 are concerned, the organisms which have played the most prominent part have been Pfeiffer's bacillus and pneumococcus.

#### BACTERIOLOGICAL EXAMINATION OF BLOOD.

##### *Blood cultures before death.*

Pneumococcus only	..	..	..	4
No organisms	..	..	..	31
Contaminated	..	..	..	2
				<hr/>
				37

##### *Blood cultures within four hours after death.*

Pneumococcus only	..	..	..	5
Pneumococcus and non-hæmolytic streptococcus				4
Pfeiffer's bacillus, mixed with other organisms				
(not determined)	..	..	..	1
Mixed cultures containing pneumococcus, non-hæmolytic streptococcus, and other organisms				
(not determined)	..	..	..	3
No organisms	..	..	..	14
				<hr/>
				27
				<hr/>

#### POST-MORTEM EXAMINATIONS.

*Accessory respiratory sinuses.*—The naked eye and microscopic post-mortem appearances of the various organs of influenza patients have been described by many workers. There is one condition, however, which does not appear to have been sufficiently dealt with, *viz.*, the presence of pus in the accessory respiratory sinuses and middle ears. Greig and Maitra(3) pointed out the importance of this observation, but few others seemed to have laid any stress on this condition. It was an extremely common finding in influenza and comparatively rare in other respiratory diseases occurring during the epidemic (see Table VIII); it is of significance too that the pus almost always contained an abundance of Pfeiffer's bacillus.

Pus in the accessory respiratory sinuses associated with the presence of Pfeiffer's bacillus is an important point in the diagnosis of influenza.

The amount of pus varies considerably as does its appearance. In many cases, especially in the sphenoidal sinuses, two or more cc. of pus can be obtained; in others the lining membrane may be only red-stained and the pus must be scraped away with a platinum loop. Sometimes it is greenish in colour, at other times hæmorrhagic, the lining membrane being usually blood-stained. In no case was there any inflammation of the dura mater or necrosis of the underlying bone.

Tables VI, VII and VIII give the results of the bacteriological examination of the sinuses in 32 cases of influenza, and in 30 cases of other respiratory diseases.

The bacterial flora in the sinuses of the influenza cases here differed somewhat from that of the Calcutta cases. In Bombay non-hæmolytic streptococci and members of the catarrhalis group occurred associated with Pfeiffer's bacillus in comparatively large number while in Calcutta pneumococcus and Pfeiffer's bacillus were always present to the almost total exclusion of other organisms.



TABLE VI.  
Bacteriological examination of the accessory respiratory sinuses in cases of influenza.

	FRONTAL SINUSES.					ETHMOIDAL SINUSES.					SPHENOIDAL SINUSES.					MIDDLE EARS.				
	Pus.	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.	Micrococcus catarrhalis.	Pus.	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.	Micrococcus catarrhalis.	Pus.	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.	Micrococcus catarrhalis.	Pus.	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.	Micrococcus catarrhalis.
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Present. — Absent. o Not examined.

TABLE VII.

*Summary of Table VI.*

No. of post mortem examinations made		39
Pus in one or more of the sinuses	Pfeiffer's bacillus	26
	Pneumococcus	26
	Streptococcus	14
	Catarrhalis group	10

Sinuses.	Pus present.	Pus examined bacteriologically.	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus.	Catarrhalis group
Sphenoidal	30	29	25	24	12	6
Ethmoidal	27	26	21	14	12	7
* Frontal	24	21	18	12	8	6
Middle Ear	15	15	14	10	7	5

\* In one case the frontal sinuses were not opened.

*Notes.*—Out of 39 cases examined the sinuses were found to be unaffected in seven only.\*

The sphenoidal sinuses were the most and the middle ears the least frequently affected.

It was usual for the majority of the sinuses to contain pus, but in one case the frontal, and in another case the sphenoidal, were the only sinuses affected.

The purulent effusion was blood-stained in 19 out of the 32 cases.

TABLE VIII.

*Bacteriological examination of the accessory respiratory sinuses in respiratory diseases other than influenza.*

	ACCESSORY RESPIRATORY SINUSES	
	Pus	
		Pfeiffer's bacillus.
		Pneumococcus.
LOBAR PNEUMONIA		
1	+	-
2	-	-
3	-	-

TABLE VIII.—*contd.*

			ACCESSORY RESPIRATORY SINUSES.	
			Pus.	
			Pneumococcus bacillus.	Pneumococcus.
LOBAR PNEUMONIA.				
4	..			
5	..	..		
6	..	..		
7	..	..		+
8	..	..		
9	..	..	+	+
10	..	..	..	
11	..	..	-	
12	..	..	-	
13	..	..		
14	..	..		
15	..	..	+	+
PULMONARY TUBERCULOSIS.				
1	..	..	-	-
2	..	..	-	-
3	..	..		
4	..	..	-	-
5	..	..	+	+
6	..	..	-	-
7	..	..	-	-
8	..	..	+	-
9	..	..	-	-
10	..	..	-	-
11	..	..	-	-
ASTHMA.				
1	..	..	-	-
2	..	..	-	-
3	..	..	-	-
4	..	..	-	-
Total No. of cases .. 30			7	7

*Lungs.*—The lesions in the lungs of influenza patients have been varied and no condition has been found which could be described as being 'typical' of influenza. Two types of lesion have, however, been most often encountered.

(1) Large, heavy swollen, oedematous lungs. The pleural cavities contain a large amount of blood-stained fluid but little or no pus, and

there is a thin filmy deposit of fibrin on the visceral pleura under which patches of hæmorrhage can be observed. On section the lungs drip with blood-stained fluid, and the surface is found to be studded with little droplets of pus which exude from the cut ends of the bronchioles. From this pus a pure or nearly pure culture of Pfeiffer's bacillus can be obtained. The bronchioles and bronchi are deeply congested and show patches of hæmorrhage. Cultures from the large bronchi are mixed, containing pneumococci, streptococci and other organisms as well as Pfeiffer's bacillus. The bronchioles are surrounded by areas of hæmorrhage but are not thickened. The intervening lung substance is soggy but still elastic; abscess formation is uncommon. Such areas of emphysema as are present are confined to the edges of the lungs. The bronchial lymph nodes are red, enlarged and soft.

(2) The second type closely resembles septic broncho-pneumonia. The areas of consolidation are usually scattered throughout both lungs and are not confined to the neighbourhood of the bronchioles. They are soft, raised, reddish-yellow, or greyish in colour often confluent and hæmorrhagic; abscess formation is common. Cultures from the bronchioles never give *pure* cultures of Pfeiffer's bacillus. There is often a well marked purulent pleural effusion with a shaggy deposit of fibrin and pus on the surface of the lung. The bronchial lymph nodes are large, soft, and often variegated with yellow patches containing pus.

TABLE IX.

*Bacteriological examination of the lungs, etc., in 39 cases of influenza.*

	LARGE BRONCHI.			BRONCHIOLES.			CONSOLIDATED PORTIONS OF LUNG.			ABSCESS OF LUNG.			PURULENT PLEURAL EFFUSION.		
	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus (non-haemolytic).	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus (non-haemolytic).	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus (non-haemolytic).	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus (non-haemolytic).	Pfeiffer's bacillus.	Pneumococcus.	Streptococcus (non-haemolytic).
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Notes:—

(1) In the cases marked ++ Pfeiffer's bacillus was obtained in pure culture.

(2) The case numbers 1 to 32 correspond to the numbers in Table VI.

(3) In the cases marked o the lesion in question (consolidated lung, abscess or purulent pleural effusion) was not present. By 'consolidated lung' is meant the soft, raised patches such as are observed in septic broncho-pneumonia.

TABLE X.

*Summary of Table IX.*

	LARGE BRONCHI.	BRONCHIOLES.	CONSOLIDATED PORTIONS OF LUNG.	ABSCESS OF LUNG.	PULMONARY INFILTRATION.
	(39 cases examined)	(39 cases examined)	(23 cases examined)	(12 cases examined)	(16 cases examined)
<i>Exudate</i>					
Pure	0	10	0	0	0
Mixed	36	26	17	2	4
Pneumococcus	27	20	15	8	10
Streptococcus <i>non-haemolytic</i>	39	26	20	12	9

Note.—In three cases a haemolytic streptococcus was isolated from the lungs.

*Agglutination tests.*—A series of agglutination tests were carried out in continuation of the work done in Calcutta.

(1) The serum of 48 influenza patients taken during the second week of the disease was tested against homologous strains. Six of these strains were spontaneously agglutinable in distilled water, normal salt solution and in the serum of healthy persons. Of the others, 30 were agglutinated by the patient's serum and not by that of healthy persons.

The agglutination titre varied from 1 in 32 to 1 in 256.

(2) The sera from these patients were tested against four heterologous strains isolated from sputa obtained from the same hospital (Dadar) and during the same period of the epidemic as the homologous strains.

Out of the 30 sera which agglutinated the homologous strains only 12 agglutinated one or more of the heterologous strains.

(3) Sixty-nine strains were tested against high titre sera prepared by injecting rabbits with the four strains mentioned above. No absorption experiments were carried out. Cross agglutination experiments did not reveal any marked evidence of specific grouping. All that can be said is that more or less identical strains do occur but the great majority of them vary greatly in their agglutinative and power to produce agglutinins.

*Summary and discussion.*

I. *The rôle of Pfeiffer's bacillus in influenza.*—This is still a matter of debate. Doubt regarding the etiological significance of this organism in influenza has been largely due to the diverse bacteriological findings in the various epidemics, by failure to reproduce the disease in animals and later on to the claims put forward by supporters of the 'filter-passer theory.' Now that this theory has suffered many reverses, attention is once more being directed to Pfeiffer's bacillus, and workers have begun to revolt against the hard and fast interpretation of the so-called Koch's postulates.

To quote from a paper by Fildes and McIntosh,(4) the first condition laid down by Koch is fulfilled if the virus is 'recognised in a large proportion of the cases of the disease preferably in relation to the chief lesions.'

This I have shown to be true for Pfeiffer's bacillus in the influenza epidemics in Calcutta and Bombay *where special attention was paid to the cultural requirements of that organism.*

To summarise my findings :—

(1) Seventy-five per cent of 175 specimens of the sputum or pharyngeal secretion of influenza patients obtained during the epidemics contained Pfeiffer's bacillus.

(2) The accessory respiratory sinuses of 41 out of 53 cases, examined post-mortem, contained pus. From this pus Pfeiffer's bacillus was recovered in 34 cases (83 per cent), and sometimes in almost pure culture.

(3) The bronchi, bronchioles, consolidated lung tissue or pleural fluid was examined in 39 cases, post-mortem. Pfeiffer's bacillus was found in 92 per cent of the cases; most frequently in the bronchi or bronchioles and in the latter situation often in pure culture.

Additional evidence of the causative rôle of Pfeiffer's bacillus in influenza is as follows :—

In the pharyngeal secretion of healthy persons obtained during the Bombay epidemic (August and September, 1919) Pfeiffer's bacillus could only be demonstrated in 18 per cent of 138 specimens, while during the same months it was found in 75 per cent of the sputa of influenza patients examined.

*Agglutination tests.*—The following conclusions have been drawn from a fairly large series of experiments with the blood serum of influenza patients, convalescents, and healthy persons, carried out in Calcutta and Bombay.

(1) The blood serum of influenza patients whose respiratory tract is infected with Pfeiffer's bacillus possesses the power of agglutinating the homologous strain (77 per cent of 81 cases).

(2) Agglutination of heterologous strains occur in 40 per cent of the cases.

(3) The serum of healthy persons does not agglutinate Pfeiffer's bacillus.

(4) Agglutinins appear in the blood of patients during the first week of the illness, and persist for at least five weeks afterwards.

The serum of 17 persons suffering from diseases other than influenza (tuberculosis, lobar pneumonia, Bright's disease, Hodgkin's disease, ankylostomiasis, kala-azar, and certain surgical conditions, *e.g.*, hernia and hydrocele) failed to agglutinate four strains of Pfeiffer's bacillus. One case of lobar pneumonia with empyema and one case of ankylostomiasis without any history of influenza gave positive reactions.

*Animal experiments.* The second and third conditions laid down by Kock, *etc.*, that the virus should be shown to be living, and should be capable of reproducing the disease in other animals, seem to have been adequately fulfilled in the work of Blake and Cecil.(5)

In the writer's experiments with mice, rabbits and guinea-pigs, no lesions characteristic of influenza were obtained by inoculation with Pfeiffer's bacillus intranasally, intraperitoneally or intravenously.

The following observations, however, may be of interest:—

(1) Pfeiffer's bacillus causes a septicæmia in white and brown mice. Intraperitoneal inoculation of mice is a useful method of recovering Pfeiffer's bacillus and pneumococcus from sputum.

(2) The presence of pneumococcus seems to increase the invading power of Pfeiffer's bacillus for mice.

(3) Intravenous injection of live cultures of Pfeiffer's bacillus in small doses does not cause a septicæmia in rabbits but renders the animal susceptible to intercurrent infections with other organisms, *e.g.*, the Pasteurella group, and staphylococcus aureus.(6)

(4) The bacillus can be recovered from the heart's blood of rabbits up to 12 hours after intravenous inoculation, but subsequently disappears from the circulation and is not found in the organs after 18 hours.

(5) The post-mortem condition of rabbits and guinea-pigs following the intravenous or intraperitoneal injection of large doses of Pfeiffer's bacillus suggests that death is due to toxæmia.

(6) A similar condition can be produced by the inoculation of saline extracts of dead bacilli repeatedly frozen and thawed and crushed in a mortar and by the products of the growth of the organism in blood broth.

Rabbits were not protected by small doses of this supposed exotoxin.

II. *The Pneumococcus*.—The evidence brought forward shows that pneumococci were a most important factor in the influenza epidemics investigated.

(1) The pneumococcus was found in 96 per cent of 124 specimens of sputum examined during the Calcutta epidemic and in 54 per cent of 141 specimens in the Bombay epidemic.

(2) Pneumococcus was present in 37 per cent of 179 blood cultures taken from influenza patients during life or within 6 hours after death.

(3) It was isolated from the pus of the accessory respiratory sinuses in 83 per cent of the cases examined post-mortem and from other parts of the respiratory tract in 72 per cent.

Certain facts seem to point to its being only a secondary invader :

(1) It was much more frequently found in the influenza cases during the comparatively severe epidemic in Calcutta than in the mild epidemic in Bombay.

(2) During the epidemic in Bombay the percentage of healthy persons carrying pneumococci was almost as great as that of the influenza patients (see Table IV).

(3) Seventy-four per cent of the strains examined serologically belonged to the heterogenous collection known as Group IV (American classification).

It was nevertheless a most potent factor in influencing the mortality rate in the epidemics investigated.

III. *The Streptococcus*.—Hæmolytic streptococci were conspicuous by their absence, and all the evidence goes to show that non-hæmolytic streptococci, although present in the great majority of the cases, were not organisms of importance and only accidentally connected with influenza.

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# STUDIES ON THE FLAGELLATES OF THE GENERA HERPETOMONAS CRITHIDIA AND RHYNCHOIDOMONAS

No. 7

SOME MISCELLANEOUS NOTES ON INSECT FLAGELLATES.

BY

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[Received for publication, January 18, 1921.]

## INTRODUCTION.

DURING the many years I have studied insect flagellates I have never succeeded in discovering one in any of the hæmatophagous species of the genus *Musca*. This is not due to any lack of material for in Madras there are several species such as *Musca pattoni*, *Musca convexifrons*, *Musca humilis* and *Musca nigrithorax*. These negative findings are somewhat remarkable when the peculiar habits and food of these flies are taken into consideration. I have, however, never had an opportunity of examining *Musca bezzii* and *Musca gibsoni* for such flagellates until I came to Coonoor; it was not long before I discovered a species of *Herpetomonas* in the former.

*Musca bezzii* is one of the largest species of the genus and is common on the Nilgiri Hills extending down to about 2,000 feet above sea level, but is most abundant higher up. Both sexes, but more particularly the females, may be seen in large numbers flitting about and settling on the bodies of cattle, scavenging for their food, which is to a large extent, either fresh, or dried blood, on the skins or hairs of cattle; also the discharge from the eyes and noses of animals. When any of the biting flies such as *Phlebotomus crassirostris*, *Stomoxys calcitrans*, and any

of the Tabanidae settle down to a meal of blood, the female *Musca bezzii* attempts to share the meal with them. It will worry one of the larger blood sucking flies, and compel it to withdraw its proboscis and will then suck up any blood which exudes. Sometimes two or even three specimens of *bezzii* may be seen worrying *Stomoxys calcitrans*, and eventually forcing it to withdraw its proboscis. *Stomoxys calcitrans* always resents these attempts vibrating its wings on the approach of *bezzii* evidently hoping in this way to drive it away; it only withdraws its proboscis after considerable irritation. When there are no biting flies about, the female *bezzii* may be seen searching among hairs on the legs and abdomen for a spot of dried blood, and when it discovers one it softens it with its salivary secretion, and then sucks it up. It is, therefore, not surprising to find blood either fresh or dried in its intestinal tract.

This habit is common to all the haematophagous species of the genus, and it can be easily understood that these flies may carry bacteria, and such protozoa as trypanosomes, from an infected to a healthy animal by contaminating any small cut with the parasites on their labella. And this possible channel of infection should always be taken into consideration when investigating outbreaks of trypanosomiasis in animals.

At present the species found in India are the only ones whose habits have been studied, but there can be little doubt that when these flies are better known, they will be found in other parts of the world; in the past they have been confused with the house fly group. Recently, when in England, I had the opportunity of studying the habits and early stages of the common European species, *Musca autumnalis* de Geer (*caecina*), and was surprised to find how little was known about it. This species is also haematophagous in habit, and may be seen in large numbers in the summer months on the bodies of cattle and horses following the biting flies particularly *Stomoxys calcitrans*. It is also common to see large numbers feeding on the secretion from the eyes and noses of these animals, and they are then troublesome pests. *Musca autumnalis* lays the rounded egg in patches of fresh cow dung dropped in the fields, and its larva can be found in large numbers in these patches; they can always be recognised by their large black posterior spiracles. They migrate from the dung and pupate in the ground some distance away. I have found living puparia in the winter in such situations clearly showing that it hibernates in this stage.

Before passing on to the *Herpetomonas* of *Musca bezzii*, I find it necessary to refer to a recent paper by Townsend in which he proposes

splitting the Linnean genus *Musca* into three genera, *Promusca* for *Musca domestica*, *Eamusca* for *Musca autumnalis*, and *Virioparomusca* for *Musca bezzii*. He states that 'for almost a century the generic name *Musca* has, by misuse, been perverted from its rightful application,' and proceeds to deal with what is considered the rightful application of the name. To say the least of it the creation of a new generic name for the cosmopolitan house fly, *Musca domestica* is a most unwise proceeding. This name has for generations been used in Medical, Zoological and Epidemiological literature, and to alter it now would lead to the utmost confusion, and distrust in systematic entomology. Fortunately Townsend's interpretation of the facts of the case do not bear close investigation, and I am indebted to Dr. Gahan, of the British Museum (Natural History) for the following notes on Townsend's new name for the house fly, *Musca domestica*.

Townsend arbitrarily assumes that Latreille (1810), was the first reviser of the genus *Musca*, whereas Lamarck nine years previously in his 'System des Vertebres,' page 310, divided the genus *Musca* into two sections, in each of which, he mentioned only one species; *Musca domestica* is mentioned in the first section, and, therefore, becomes the type of the genus. That Lamarck intended the specimen as the type is shewn by the statement on the page of the advertisement to the Volume referred to in para. 2. Moreover in saying that Latreille's designation of the type of the genus cannot be consistently set aside, Townsend has overlooked the fact that five years earlier, Fabricius in his 'System Antliatorum,' (1805), page 284, had indicated *Musca carnaria* L., as the type of the genus *Musca* by the method by which the late Mr. Kirjaldy originally drew attention, *viz.*, by giving a description of the genus under the heading of that species. Medical men, Epidemiologists and Sanitarians can, therefore, continue to use the familiar and correct generic name *Musca* for the well known house fly without feeling that they are in any way transgressing the rules of Zoological Nomenclature.

#### *Herpetomonas Craggii* Sp. Nov.

This *Herpetomonas* is parasitic in the alimentary tract of the adults of *Musca bezzii*. Although I have examined about 300 flies, I have only once succeeded in finding the adult flagellate stage in large numbers in one female fly; in a few others one or two flagellates were found in the midgut. The postflagellate stage on the other hand is quite easily found in the hindgut and rectum of about 75%. The mature flagellate



PLATE XVII.

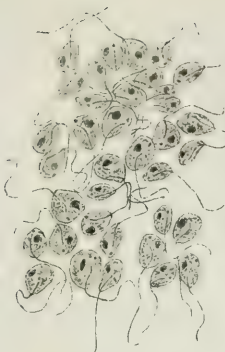


FIG. 1

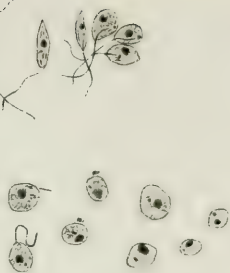


FIG. 3



FIG. 2

FIG. 5



FIG. 8



FIG. 9



FIG. 7



FIG. 4

# EXPLANATION OF PLATE XVII.

- Fig 1 A group of the round and oval postflagellates of *Herposomna* caryops from the rectum of *Musca domestica* about 800 times.
- 2 Note the long free flagella and the cellular portion.
- 3 Another group of the same about 800 times.
- 4 The final postflagellate stage about 800 times.
- 5 A rosette of four mature flagellates of *Herposomna* unipapula about 800 times.
- 6 A mature flagellate of the same with a very long thin posterior end about 2000 times.
- 7 Another adult flagellate of the same with an even longer posterior end and about 2000 times.
- 8 Another adult flagellate of the same with a shorter posterior end about 2000 times.
- 9 A mature flagellate of the same dividing. Note the posterior end has broken off about 2000 times.
- 10 Another flagellate of the same dividing about 2000 times.

# EXPLANATION OF PLATE XVII.

- Fig. 1. A group of the round and oval postflagellates of *Herpetomonas* *craggi* from the rectum of *Musca bezzii*.  $\times$  about 800 times.  
Note the long free flagella, and the cellular portion.
- .. 2. Another group of the same.  $\times$  about 800 times.
- .. 3. The final postflagellate stage.  $\times$  about 800 times.
- .. 4. A rosette of long mature flagellates of *Herpetomonas mirabilis*.  
 $\times$  about 800 times.
- .. 5. A mature flagellate of the same with a very long thin posterior end.  
 $\times$  about 2000 times.
- .. 6. Another adult flagellate of the same with an even longer posterior end.  $\times$  about 2000 times.
- .. 7. Another adult flagellate of the same with a shorter posterior end.  
 $\times$  about 2000 times.
- .. 8. A mature flagellate of the same dividing. Note the posterior end has broken off.  $\times$  about 2000 times.
- .. 9. Another flagellate of the same dividing.  $\times$  about 2000 times.

measures from  $20\mu$  to  $25\mu$  in length and from  $1.5\mu$  to  $2\mu$  in breadth. The nucleus is large and usually situated about the middle; the blepharoplast is relatively small and lies close to the anterior end. The flagellum is long and stout. These forms evidently pass down the alimentary tract and collect in the hindgut where they round up.

In its postflagellate stage the parasite can be found in large numbers rounding up rarely in the hindgut and most commonly in the rectum. These stages are shewn in Figs. 1 and 2 on Plate XVII; they are oval in shape measuring from  $2\mu$  to  $3\mu$  in length. The nucleus stains well and usually lies about the centre of the body, and at this stage the blepharoplast is a small deeply staining rod lying close beside it. In Fig. 3, Plate XVII, the final round postflagellate stage is illustrated. Some of these shew the flagellum merely attached to the margin of the body of the parasite, the cellular portion having disappeared; in others the flagellum is seen as a mere tag. The final stage varies much in size and may be as small as  $1.5\mu$  in length, the larger forms evidently divide into the smaller ones. This stage is best seen in the lower end of the rectum where they may be found in immense numbers packed together and then the rectum has a characteristic pearly white colour, is very brittle and readily tears.

It will be remembered that *Musca bezzii* is larviparous in habit, depositing one larva at a time in patches of fresh cow dung. I have examined the alimentary tracts of many larvæ, both immature and mature, but have never found the parasites in them. This *Herpetomonas* evidently passes its complete life history in the adult fly.

I have much pleasure in naming this species after Major Cragg (F.M.S.) who collaborated with me in describing the host, *Musca bezzii*.

It is interesting to note that a large percentage of the females, more than 30%, are infected with a yeast which is commonly found in clumps in the body cavity and also in the alimentary tract. About 5% of the females are infected with a species of *Habronema* which almost invariably destroys the ovaries. All the stages of this nematode may be found in the same fly. Yet in spite of these various parasites *Musca bezzii* is an abundant species wherever it occurs.

#### *Herpetomonas mirabilis* Roubaud.

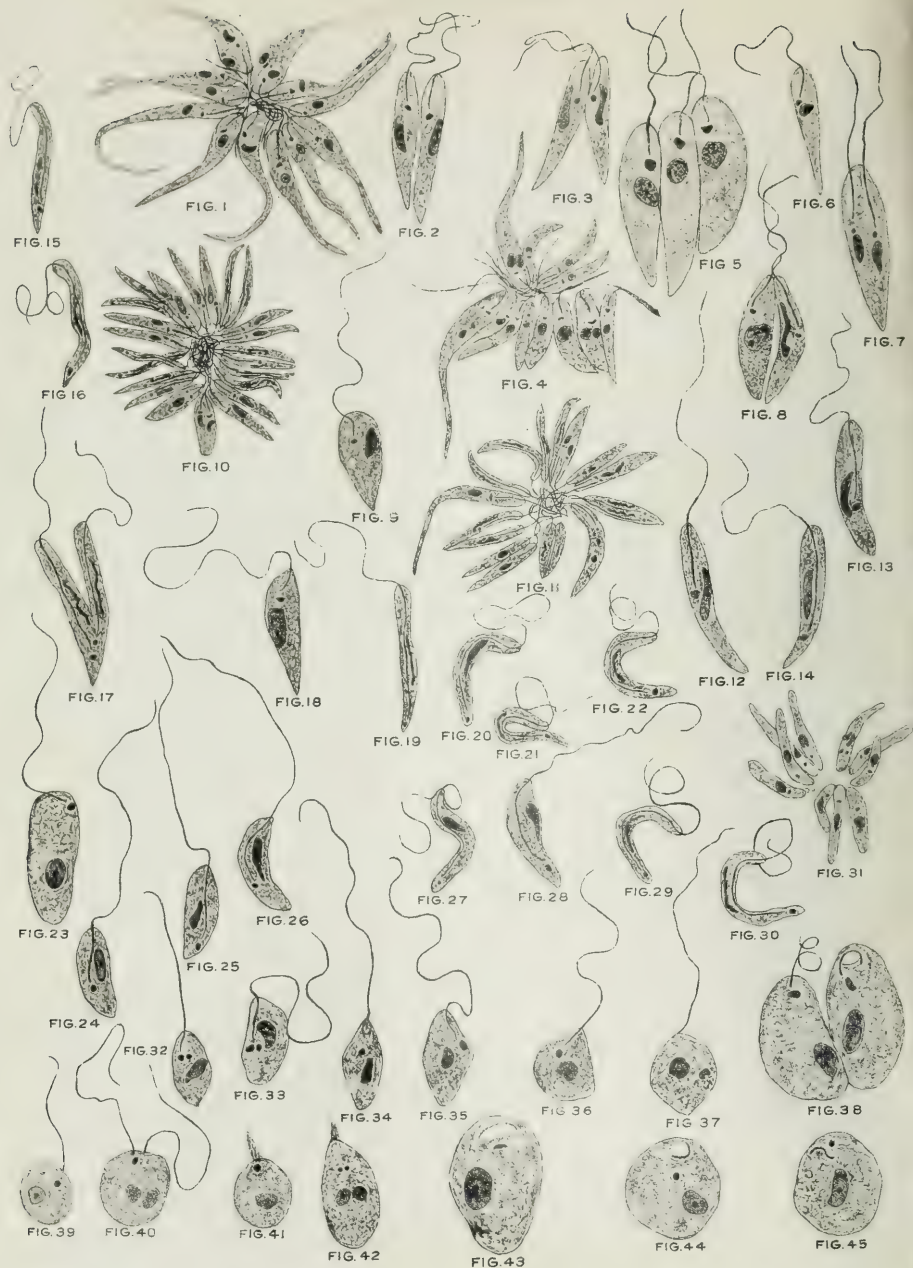
I first discovered this species in the alimentary tract of *Lucilia atripunctata* in 1906 at Madras, a large percentage of the flies caught in the bazaars being infected. Since then Roubaud has described it

from the alimentary tract of *Chrysomya albiceps* (putoria) from West Africa naming it *mirabilis*. I have also found *mirabilis* in a number of other Calliphorinae, such as *Chrysomya megacephala*, *C. albiceps* and *Lucilia cragii*. In 1913 and again in 1920, I worked out its complete life history. It was found that it readily develops in the alimentary tract of the larva of *Lucilia argyricephala*, the infection being carried over to the adult stage through the pupa.

A large number of adult flies were collected from the bazaar shops and were placed in a cage. A piece of sheep's spleen which had been flamed was pulped up and placed on a sterile watch glass and suspended in the cage; the flies readily fed on the spleen juice which was kept moist with sterile saline solution. Some of the females laid their eggs on the spleen pulp, and as soon as the larvæ had hatched out the watch glass with the spleen was removed and suspended in another cage. The larvæ were now dissected at regular intervals, and it was found that many of them contained the round stages in the upper part of their midguts, and by examining a large number it was possible to observe the round flagellates developing into the elongated ones. This process was a rapid one and about the third to the fourth day most of the larvæ contained mature flagellates. These multiplied and when the larva became matured and stopped feeding, they collected down near the openings of the Malpighian tubes, though they never entered them. On examining pupæ of various stages, it was quite easy to find masses of the flagellates in the small greenish mass of food stuff remaining over from the larval gut. A group of these forms is shewn in Plate XVII, Fig. 4. They represent the mature flagellate and it will be noted that the posterior end is attenuated and much elongated and that the nucleus lies in the broad end and the blepharoplast which is a somewhat small rod lies a little distance anterior to it. The anterior end is rounded and the flagellum is as a rule very short though it may be long. These flagellates exhibit peculiar slow waving movements, the long drawn out posterior end swaying about; it may quite well be mistaken for the flagellum in the fresh condition. It is common to find immense numbers in the hindgut just near the openings of the Malpighian tubes; their further development is very easily studied. The constant waving movements appear to draw out the posterior end so that it becomes more and more attenuated and is then seen to be attached to the body of the parasite by a slender band of protoplasm. This appearance is well shewn in Plate XVII, Figs. 5 and 6m. In Fig. 7, Plate XVII, one of these stout flagellates is shewn



# PLATE XVIII.



- Fig. 1 A rosette of mature flagellates of *W. novae-hollandiae* about 1000 times.
- Figs. 2 and 3 A part of flagellates from a rosette as seen in Fig. 1. The long posterior end has broken away about 1000 times.
- Fig. 4 A rosette of adult flagellates. Some still have long posterior ends which in most have broken off about 1000 times.
- 5 Three flagellates from a similar rosette just about to divide about 2000 times.
- 6 A single flagellate from a similar rosette about 1000 times.
- 7 A flagellate from a rosette about to divide longitudinally about 1000 times.
- 8 Further stage in the division of a similar flagellate. Note that the daughter cell on the right has an elongated nucleus and the phlebotomus is passing behind it about 1000 times.
- 9 A young flagellate from a rosette such as that seen in Fig. 11 about 1000 times.
- Figs. 10 and 11 Two rosettes of flagellates dividing and becoming more numerous.
- Note the nuclei are elongated and the phlebotomus are passing behind them about 1000 times.
- 12 13 and 14 Flagellates from such a rosette showing the changes mentioned above. The nuclei are becoming elongated and the phlebotomus are passing behind them about 1000 times.
- 15 16 and 17 Flagellates in which this change is completed. That illustrated in Fig. 17 is dividing at this stage. This is the "cystic" stage about 1000 times.
- Fig. 18 A young flagellate from a rosette such as that shown in Fig. 10 about 1000 times.
- Figs. 19 20 21 22 23 24 25 26 27 28 and 29 Cystic stage preparatory to the formation of the postflagellate.
- Note the elongated nuclei posteriorly placed phlebotomus and the long portion of the flagellum attached to the end of the cell. These forms are very like some pathogenic trypanosomes about 1000 times.
- Figs. 30 31 32 and 33 The commencement of the postflagellate stage. The cilia are shorter and eventually round up about 1000 times.
- Fig. 34 A group of flagellates feeding about 1000 times.
- Fig. 35 The formation of the postflagellate. The parasite round up and the flagellum is now only attached to the margin and division now takes place about 1000 times.
- Figs. 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 The flagellum breaks off and the parasite round up about 1000 times.

# EXPLANATIONS OF PLATE XVIII.

- Fig. 1. A rosette of mature flagellates of *H. mirabilis*. . about 1000 times.
- Figs 2 and 3. A pair of flagellates from a rosette as seen in Fig. 4. The long posterior end has broken away. . about 1000 times.
- Fig. 4. A rosette of adult flagellates. Some still have long posterior ends, while in most they have broken off. . about 1000 times.
- .. 5. Three flagellates from a similar rosette just about to divide. × about 2000 times.
- .. 6. A single flagellate from a similar rosette. . about 1000 times.
- .. 7. A flagellate from a rosette about to divide longitudinally. × about 1000 times.
- .. 8. Further stage in the division of a similar flagellate.  
Note that the daughter cell on the right has an elongated nucleus and the blepharoplast is passing behind it. . about 1000 times.
9. A young flagellate from a rosette such as that seen in Fig. 11. × about 1000 times.
- Figs. 10. and 11. Two rosettes of flagellates dividing and becoming more attenuated.  
Note the nuclei are elongated and the blepharoplasts are passing behind them. × about 1000 times.
- .. 12. 13 and 14. Flagellates from such a rosette shewing the changes mentioned above. The nuclei are becoming elongated and the blepharoplasts are passing behind them. . about 1000 times.
- .. 15. 16 and 17. Flagellates in which this change is completed. That illustrated in Fig. 17 is dividing at this stage. This is the *Crithidia* stage. . about 1000 times.
- Fig. 18. A young flagellate from a rosette such as that shewn in Fig. 10. × about 1000 times.
- Figs. 19. 20. 21. 22. 27. 29. and 30. *Crithidia* stage preparatory to the formation of the postflagellate.  
Note the elongated nuclei, posteriorly placed blepharoplast, and the long portion of the flagellum attached to the body of the cell. These forms are very like some pathogenic trypanosomes. × about 1000 times.
- Figs. 23. 24. 25. and 26. The commencement of the postflagellate stage.  
The parasites shorten and eventually round up. × about 1000 times.
- Fig. 31. A group of flagellates dividing. × about 1000 times.
- Figs. 32 to 40. The formation of the postflagellate. The parasite round up, the flagellum is then only attached to the margin and division now takes place. . about 1000 times.
- Figs. 41 to 45. The final postflagellates. The flagellum breaks off and the cell rounds up. × about 1000 times.

with the posterior end broadly attached to the body of the parasite; this portion stains a lighter colour than the rest of the parasite and appears to represent the drawn out ectoplasmic sheath. In Fig. 8, Plate XVII one of these parasites is dividing and the posterior end of the parent cell has broken off, and is lying bent near them. Fig. 9, Plate XVII shows a younger flagellate before the posterior end is drawn out to any great extent. It will be noted that in most of these long flagellates, the flagellum is broken off and that only a small intracellular portion exists. These long flagellates are always seen in groups forming large rosettes, the posterior end waving about; if they are watched for some time in the fresh condition, the posterior ends of many will be seen to become detached.

Plate XVIII, Fig. 1 shews a rosette of these elongated flagellates undergoing division; all the flagella are directed inwards, and form a tangled mass; when seen in the fresh condition they cause the parasites to wave about from side to side. When the elongated posterior end of the parasite breaks off, smaller stout flagellates result, some of these are shewn in Figs. 2, 3, 5, 6, 7, 9, 18 and 31, Plate XVIII. In these forms although the blepharoplast is usually near the nucleus, it may be seen low down near the anterior end of the parasite. By further division while still attached in rosettes, they become more and more attenuated, and often smaller in size (Figs. 4, 10 and 11, Plate XVIII). A remarkable change now takes place, the nucleus becomes more elongated, and the blepharoplast passes back towards the posterior end of the parasite. All the changes in this stage can be observed in both fresh and stained smears, these changes are well illustrated in Figs. 8, 10, 11, 12, 13 and 14, Plate XVIII. In Fig. 10, Plate XVIII, a rosette containing some of these forms with the blepharoplast at the posterior end is seen together with others in which this process is beginning. It will be noted that the nucleus at this time becomes elongated and when stained appears as a dark strip, sometimes with a wavy outline. Figs. 15, 16, 17, 19, 20, 21, 22, 27, 29 and 30, Plate XVIII, illustrate different types of this crithidia phase of *Herpetomonas mirabilis*. If this stage were examined by itself and not in relation with the previous stages, there is little doubt that it would be placed in the genus *Trypanosoma*, or at least in the genus *Crithidia*. It emphasises what I have already pointed out on many occasions, that these *Herpetomonas* exhibit marked pleomorphism in their various stages, and that unless the adult or mature flagellate

is studied, they may be confused with flagellates belonging to other and distinct genera.

These crithidia-like forms now collect in large numbers in the hind-gut of *Lucilia argyricephala*, where they round up. These rounding up forms are illustrated in Figs. 23, 24, 25, 26, Plate XVIII and it will be noted that as the parasite becomes round, the cellular portion of the flagellum becomes shortened, and later it comes to be attached only to the margin of the cell eventually breaking off, when the postflagellate stage is completed. These changes are illustrated in Figs. 32 to 45, Plate XVIII. The round postflagellates may be found in large numbers attached to the whole of the wall of the hind intestine, which then has a glistening white appearance, it is very brittle and will readily break. It is these forms which are ingested by the first stage larvæ in whose midguts they at once flagellate. There can, however, be very little doubt that the adult *Lucilia argyricephala* ingests these round stages when feeding on meat and sweets in the bazar shops, and that they develop in their midguts as well.

In connection with this *Herpetomonas* of the Calliphorinæ, it is interesting to note that it cannot live in the alimentary tract of *Musca nebulosa*. I have carried out some exact transmission experiments which have clearly demonstrated this fact. In India I have only found *Herpetomonas mirabilis* in the Calliphorinæ. *Herpetomonas muscæ domesticæ* on the other hand will readily live and multiply in the alimentary tract of many of the Calliphorinæ. As a result of these and other observations on the Herpetomonads of coprophagous and necrophagous flies, I believe that species found in different closely related Diptera are distinct, and not identical with those found in other species. Some observers would include all the Herpetomonads of necrophagous and coprophagous Diptera under the generic name *Herpetomonas muscæ domesticæ*; transmission experiments do not support this view.

I have succeeded in obtaining a pure culture of *Herpetomonas mirabilis* in the NNN medium, but it does not flourish in it and requires to be constantly subcultured.

*Herpetomonas muscæ domesticæ* Burnett.

I have found this *Herpetomonas* parasitic in the following species of Diptera: *Musca nebulosa*, *Musca humilis*, *Fannia canicularis*, *Borborus* sp., *Drosophila* sp., *Lucilia argyricephala*, *Lucilia craggi*, and in several other Calliphorinæ. In all it is quite common to find the mature flagellate

stage in the peritrophic membrane in an active stage of division, and then it will be noted that all the parasites exhibit that appearance which Prowazek long ago mistook for a biflagellate. I have pointed out that *Herpetomonas muscae domesticae* only has one flagellum, and that these forms with a double flagellum only represent a process of simple division. If these parasites are watched long enough in the fresh condition, they will be seen to divide into two and the resulting parasites then only possess one flagellum, but soon themselves begin to divide again and shew the biflagellate appearance.

*Herpetomonas muscae domesticae* though it is readily acquired by adult flies when they feed on the excreta of others containing the parasite, it can also live and multiply in the alimentary tract of the larvae of some of its insect hosts. I have recently found that the larvae of *Musca nebula* are commonly infected with this flagellate, the infection then being carried over to the nymph and the adults when they hatch out are infected. It will be easily understood how this infection is acquired, for it is only necessary to watch a heap of horse manure to see large numbers of females of *Musca nebula* crawling over it, and when laying their eggs in it pass out the round stages of the parasite on the surface. The young larvae come up to the surface at dusk to feed and then ingest the parasite.

*Herpetomonas muscae domesticae* grows very luxuriantly in the NNN medium, but it is extremely difficult to obtain a pure culture owing to the many bacteria, which are present in the alimentary tract of all coprophagous and necrophagous flies. I have, however, succeeded in obtaining a pure culture by dissecting out the peritrophic membrane of *Larida argyricephala* containing the flagellate stage and transferring some of the contained flagellates to the NNN medium; in this particular instance there did not happen to be any bacteria with the flagellates.

This *Herpetomonas* like all others cannot live in the NNN medium with bacteria as they soon overgrow it and kill it. But in the alimentary tract of its insect host it can live with faecal bacteria as long as the bacteria do not gain the ascendancy. But this sometimes happens and then it will be noted that almost the whole of the hindgut is lined with a particular species of bacterium, and when this is found no flagellates can be seen in this part of the alimentary tract. There seems to be no doubt, therefore, that many of these flagellates can live in association with bacteria with bacteria in the alimentary tracts of their hosts, but that in the

majority the bacteria never overrun the whole of the alimentary tract, thus enabling the flagellates to maintain themselves.

*Herpetomonas Sarcophagæ* Prowazek.

This species of *Herpetomonas* is very common in many species of *Sarcophagæ*, and in the majority of specimens examined it will be found in the hindgut in the round postflagellate stage. But if a large number are examined, in a few the mature flagellates will be found in the midgut. It would appear that the flies become infected early in their life histories, and that the specimens which are usually examined are those which are ready to larviposit, a considerable time has then elapsed since the infection was acquired and the parasite has completed its cycle of development. I have carried out a number of transmission experiments with this *Herpetomonas* from time to time, in order to see whether this species would live in the alimentary tract of the house fly, *Musca nebulo*, but I have always failed in infecting it. This seems to indicate that *Herpetomonas sarcophagæ* is a distinct, though close ally of *Herpetomonas muscæ domesticæ*. It is, however, very similar in structure and has a large blepharoplast and a thick flagellum, and belongs to the same group.

*The Flagellates of Sepsids.*

Many years ago in Madras I discovered a very striking species of *Crithidia* in a species of *Sepsid*, which was very common on the heap of cow dung kept in the Sewage Farm of the King Institute. This parasite was subsequently found in another species of the same genus of dung flies, and in this there was also a species of *Herpetomonas*. Both these parasites are illustrated on Plate XIX. The *Crithidia* (Figs. 1, 2, 3, 4 and 5, Plate XIX) exhibits some peculiarities common to certain species of the genus *Trypanosoma*. The flagellum is short and runs along the margin of the body in an undulating manner to the blepharoplast which is situated as a rule near or behind the nucleus, but may be during the process of division anterior to it. It is of interest to note that this *Crithidia* is parasitic in the alimentary tract of a purely dung feeding insect and can under no circumstance be acquired from the blood of a vertebrate.

The *Herpetomonas* (Figs. 6 to 10, Plate XIX) was common in one species of *Sepsis* along with the *Crithidia*, but there was no doubt that they are distinct species for the *Crithidia* was found in the alimentary tract of another *Sepsis* without the *Herpetomonas*. It is a strikingly distinct species with a relatively short flagellum and large blepharoplast.

EXPLANATION OF PLATE XIX.

- Figs. 1 to 5. The *Crithidia* sp., from the alimentary tract of a Sepsid.  
Note the position of the blepharoplast, the short free flagellum  
and the evidence of an undulating membrane shown in Figs. 2  
and 3. These flagellates have a trypanosomic facies.  $\times$  about  
1500 times.
- Figs. 6 to 10. The *Herpetomonas* sp., from the alimentary tract of a Sepsid.  
Note the large size of the blepharoplast and the relatively  
short flagellum.  $\times$  about 1500 times.

# EXPLANATION OF PLATE XIX.

Figs. 1 to 5. The *Cystodex* sp. from the alimentary tract of a Sepiid.  
 Note the position of the pleuropharynx, the short free flagellum  
 and the evidence of an undulating membrane shown in Figs. 2  
 and 3. These flagellates have a typanosomic facies. About  
 1500 times.

Figs. 6 to 10. The *Apicomonax* sp. from the alimentary tract of a Sepiid.  
 Note the large size of the pleuropharynx and the relatively  
 short flagellum. About 1500 times.

# PLATE XIX.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.



FIG. 7.



FIG. 8.



FIG. 9.



FIG. 10.



In concluding these miscellaneous notes on some insect flagellates, I would like to point out that there is a very large field for original work on these parasites in India, and the material is unlimited. I trust those who have opportunities of studying these parasites will do so, for I am convinced that careful studies of their life histories would yield much information which would help us to understand those related forms, which are, perhaps, on the way to become pathogenic to man and animals. Transmission experiments on white mice would give us some valuable information as to whether the species found in India are capable of living in the tissues of the smaller laboratory animals. I would, however, warn the observer who undertakes such experiments that the white Japanese performing mice, which are usually sold in India, seem to be of little use for such experiments. I have inoculated many of these mice with several species of *Herpetomonas* including *Herpetomonas downsi*, but have never yet succeeded in obtaining any positive results.

STUDIES ON THE FLAGELLATES OF THE  
GENERA HERPETOMONAS, CRITHIDIA  
AND RHYNCHOIDOMONAS.

No. 8.

NOTE ON THE BEHAVIOUR OF *HERPETOMONAS TROPICA*  
WRIGHT, THE PARASITE OF CUTANEOUS HERPE-  
TOMONAS (ORIENTAL SORE) IN THE BED BUG  
*CIMEX HEMIPTERA FABR.*

BY

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[Received for publication, January 27, 1921.]

IN an earlier paper the senior author recorded some experiments which were carried out at Cambay with the object of discovering the method of transmission of the parasite of Oriental Sore; the following is a short summary of the experiments.

*Experiment 1.*—An attempt was made to try and infect cuts and scratches on the human skin by allowing house flies (*Musca nebulosa*) to settle on them after they had contaminated their legs and proboscides with the characteristic parasite laden white discharge from a sore. The results were entirely negative.

*Experiment 2.*—Dissection of the alimentary tracts of house flies which had fed on the discharge mentioned above conclusively proved that the parasite disappears from the midgut of the fly in six hours, and that it never reaches the hindgut and rectum in a living condition. The parasite does not undergo any developmental changes in the alimentary tract of the house fly. These two experiments although carried out under the most favourable conditions left no doubt that the house fly plays no part in the transmission of the parasite from man to man.

*Experiment 3.*—Body and head lice although allowed to crawl over and feed at the margin of a sore never become infected, and all the evidence on the etiology of the disease is opposed to the view that lice play any part in the transmission of the parasite in nature.

*Experiment 4.*—No evidence could be found to incriminate any biting fly as being the carrier of the parasite. It is true *Phlebotomus manitus* occurs in Bombay but in such small numbers that it was not possible to carry out any experiments with this fly. Its probable relation to the spread of the parasite will be fully discussed in another paper by the senior author.

In view of the hypothesis that fleas (*Pulex irritans* and *Ctenocephalus canis*) are believed to be the transmitters of the parasites of human and canine kala-azar along the Mediterranean littoral, it was necessary to consider whether fleas could be concerned in the transmission of the parasite of Oriental Sore. Although no feeding experiments were carried out with any of these insects, no evidence could be found to incriminate them, and no case of Oriental Sore was seen in a dog. Feeding experiments with fleas are difficult to carry out, and further it should be remembered that *Pulex irritans*, *Ctenocephalus canis* and *C. felis* are infected with their own natural parasites which may be confused with any they may ingest.

*Experiment 5.*—A large number of experiments were carried out with the bed bug *Cimex hemiptera* fed at the margin of the sore and at some distance from it, and the following conclusions were arrived at.

As the parasite of Oriental Sore occurs in the peripheral blood, it can be ingested by the bug, in which it develops into its flagellate stage but only if the temperature is below 25°C. The flagellates were not seen in stained smears of the alimentary tract of the adult bug after the tenth day, and it was believed that the parasite dies out in the adult, but remains a longer time in the alimentary tract of the nymph. The fact that Oriental Sore only occurs in those parts of India where there is a

decided cold weather supports the view, that temperature plays an important part in the development of the parasite in the insect host, and the parasite will only flagellate in the bug when the temperature is below 25°C.

As a result of these observations, the conclusion was reached that in Cambay, *Cimex hemiptera* appeared to be the only possible insect carrier. Feeding experiments with infected bugs, however, conclusively proved that the parasite is not transmitted by the bug in the act of sucking blood.

From some observations on Oriental Sore in Mesopotamia, which were recorded in a recent note, the senior author came to the conclusion that an allied parasite in that country found its way into the skin when one or other of the species of *Phlebotomus* infected with *Herpetomonas phlebotomi* Mackie was crushed in the act of biting. In other words that the parasite of Oriental Sore in Mesopotamia is in reality *Herpetomonas phlebotomi* which on multiplying in the skin produces the well-known sore.

With this hypothesis in view, we have carried out a long series of feeding experiments by feeding *Cimex hemiptera* on cultures of the parasite of Oriental Sore, which Dr. Row very kindly placed at our disposal, and also from cultures of a sore produced experimentally on the arm and leg of one of the Laboratory Assistants, Motha, Pasteur Institute of Southern India, specially sent to Jodhpore for the purpose. Dr. Row tells us that his culture was originally obtained from a typical case of Oriental Sore contracted in Cambay, and that he has kept it alive and virulent by passing it through animals and numerous subcultures. In this paper we propose recording our observations on the behaviour of the parasite in the alimentary tract of the bug, *Cimex hemiptera*, and at the same time reviewing some points in the etiology of the disease.

#### MATERIAL AND METHODS.

It will be remembered that one of us in collaboration with Cornwall devised a simple and most successful method of feeding bugs on cultures of the parasite of kala-azar through a piece of fresh rabbit's skin. The technique involved was fully described at the time, and we are surprised that no other observers, who have opportunities of working at the kala-azar problem, have carried out similar experiments. In order to emphasise the great utility of this method of feeding bugs, and for the

matter of that any other blood-sucking insects, we will again describe the technique in detail, and some modifications we have introduced.

In the case of the parasite of Oriental Sore, it was found that a culture six days old gave the best results. The following material and apparatus is required:

1. About 8 to 12 tubes of NNN medium inoculated with the parasite six days previously.

2. Small glass cylinders varying in length from 4 to 8 cm. in length, plugged at both ends with cotton wool and autoclaved; these cylinders are made by cutting off the ends of test tubes.

3. A young rabbit with the hair on the abdomen cut close to the skin.

4. Sterile dissecting instruments, such as forceps, scissors and knives.

5. Plastecene.

6. Tubes of starved bugs in all stages. The insects being allowed to rest on a long piece of filter paper a narrower piece placed at the bottom of the tube to allow any which happen to fall down to regain the longer piece of filter paper.

7. Fresh rabbit's blood mixed with a small quantity of sterile citrate. It is important to make quite certain that the blood is taken from a healthy animal, blood from a rabbit suffering from coccidiosis is fatal to the bugs and the parasites alike.

One observer now prepares the feeding mixture by drawing up the water of condensation from the culture tubes under strictly sterile conditions, placing it in a sterile glass vessel. A fixed quantity of rabbit's blood is now added to it and the fluid is thoroughly mixed. A loopful is examined in a hanging drop in order to note the condition and number of the parasites. Another observer now proceeds to remove the skin of the rabbit which is first killed by an air embolus. The skin on one side is rapidly reflected, and an assistant then places the flanged end of the prepared cylinder, having first removed the cotton plug against the skin which is tied over the tube with some thread. The skin is now cut away round the tube, and in this way the ends of as many tubes as are required are covered with skin, the hairy surface being outermost. The tubes are now fixed to the test tubes containing the bugs, first being careful to draw up the filter paper to the mouth of the tube; the bugs now have free access to the hairy side of the skin. The feeding tube is now pipetted into the other end of the tube with sterile precaution, and the cotton

plug replaced. It is important to disturb the bugs before arranging the feeding tube, and this is best done by transferring them from the tube they have been kept into another similar one; they will begin feeding all the sooner if this is done. The tube is now fixed to the test tube containing the bugs by strips of plastecene. The whole arrangement is shown in the text figure



After having dissected a large number of bugs fed on cultures of *H. tropica* and *H. donovani* in the way described above, we have found that in a certain percentage, often a very large percentage, contained dead parasites in their midguts. For a long time we failed to discover the reason for this until it occurred to us that the bugs had actually sucked up dead parasites from the cultures, and that they had not died on entering their midguts. In preparing the culture fluid it will be remembered that all the water of condensation is drawn up from the tubes and then mixed with the rabbit's blood. On examining this fluid we often noted that there were a large number of dead parasites. Now if the feeding fluid is not well shaken up when the bugs are feeding the dead parasites sink to the bottom, and naturally they are the first to be ingested by the bugs when they insert their proboscides through the skin. Even with vigorous and continuous shaking, while the bugs are feeding, they cannot but help sucking up a large number of dead flagellates which are always tending to sink to the bottom of the tube on the side of the skin nearest to the bugs. In order to get over this difficulty, we have gradually evolved a different and much more satisfactory method of growing the parasites. About 48 hours after the culture tubes for the intended feeding experiment are sown, they are sloped so that the fluid of condensation containing the young growing flagellates spreads over the blood agar slope, and when all the fluid has evaporated, the parasites will be found growing luxuriantly on the agar in minute colonies which are clearly visible when in large numbers as a moist film. It is only necessary to take a light scraping off the surface of the agar, and to place it in a drop of saline to note that the parasites are in a vigorous condition, exhibiting all the various changes in growth and multiplication; and more important still there are not nearly so

many dead flagellates. When the growth on the agar is found to be at its optimum point, and has covered almost the whole surface of the agar slope, all the fluid of condensation having evaporated, a fixed quantity of human blood mixed with some sterile sodium citrate is run into the tubes, and with a platinum loop the colonies are lightly scraped off the surface of the agar and mixed with the blood. The mixture is then pipetted up and placed in the feeding tube as described above. By this modification of the original technique we have been able to infect bugs with many more living flagellates. As the room temperature in Coonoor is at all times well below 25°C, there was no necessity of placing the bugs in a cold incubator.

A large number of bugs were infected and one or more were examined every day. Each bug was dissected in a drop of saline. The midgut on being severed from the oesophagus and hind intestine was transferred to another drop of saline and opened to allow the contents to escape into the saline. This was then examined under a 1-6th objective in the fresh condition and the number and condition of the motile flagellates noted; the same was done in the case of the hind intestine and rectum. Each drop of saline containing the contents of the various parts of the alimentary tract were lightly smeared out on the slides with a small piece of coverslip fixed in absolute alcohol and then stained with Romanowsky's stain. It was noted that only the active flagellates could be recognised in the fresh condition and that most of the smaller forms and particularly those without flagella could not be detected with certainty; these are best seen in stained preparations. This is particularly so in the case of the contents of the hindgut and rectum which always contain a black granular fluid which obscures everything. Particular care was taken to examine the Malpighian tubes and the salivary glands, but we have never been able to find any parasites in these organs.

#### THE BEHAVIOUR OF THE PARASITE IN THE ADULT BUG.

A large number of adult bugs were fed on a culture of *H. tropicum* in the way described above, and were not re-fed.

Bug 1. Dissected 48 hours after the feed. The midgut contents in the fresh condition contained unchanged red blood cells and a large number of highly motile flagellates particularly long sickle shaped forms which swam in the fluid with great rapidity. There were also many clumps of what appeared to be dividing forms and many motionless

parasites which were regarded as dead. The hind intestine and rectal contents showed a few active flagellates and it is interesting to note that the parasite reaches the rectum after an interval of 24 hours.

Figures 1 to 33 on Plate XX, illustrate forms of the parasites seen in the stained smear of the contents of the midgut. An examination of the figures shews that in addition to the long sickle-shaped flagellates referred to above there are round forms with short flagella and some without any flagella. Many of the parasites were undergoing division.

Figures 1, 2, 3 and 6, Plate XX, illustrate some of the round flagellates with short flagella; Figs. 4 and 5, Plate XX, round or oval forms without any flagella; Figs. 7, 8, 9, 10 and 33, Plate XX short forms in active stages of division, and Figs. 13, 14, 15, 17, 18, 19, 26 and 29, Plate XX, longer forms undergoing the same process. Fig. 22, Plate XX, illustrates one of the long sickle-shaped flagellates which are extremely motile swimming backwards and forwards in the fluid contents.

Figures 35 to 48, Plate XX, inclusive, illustrate some of the flagellates seen in the stained smear of the hind intestine, and it will be noted that in addition to the long forms there were round and oval forms and small flagellates some of which are dividing. In Figs. 49 to 54, Plate XX are shown some of the flagellates seen in the smear of the rectum. Although there were many long active flagellates which are not illustrated, the round and oval and small forms shown in the drawings were only seen in the stained smear, owing to the difficulty in detecting these forms among the granular debris of the rectum. It is interesting to note that the parasite reaches the rectum of the bug in 24 hours and that there the parasites may be seen in process of active division.

Bug 2. *Dissected 4 days after the original feed on the culture.* The midgut was almost empty, and in the fresh condition the contents shewed a large number of highly motile flagellates, and particularly many long sickle-shaped forms. Figs. 55 to 65, Plate XX, illustrate some of these flagellates as seen in the stained smear. Fig. 62 shows a group of short flagellates in all stages of binary fission, and Fig. 63, Plate XX, shows two parasites which though not yet separated are again dividing.

The hind intestine contents showed a large number of active flagellates, the long sickle-shaped forms particularly predominating. The rectal contents similarly contained a large number of these forms some of which are illustrated in Figs. 66 to 74, Plate XX.



*Herpetomonas trophica*



Bug 3. *Dissected 6 days after the original feed on the culture.* The midgut contained a few, the hindgut and rectum many, motionless flagellates which were apparently dead. It is quite common to find this condition in bugs fed on cultures of the parasites and the only explanation we can offer is that these bugs had sucked up dead parasites.

Bug 4. *Dissected 6 days after the original feed on the culture.* The midgut was empty and when teased up in the saline and examined in the fresh condition, a few active flagellates and some dead ones were seen. Some of the forms seen in the stained smear are shown in Figs. 75 to 78, Plate XX. Fig. 75, Plate XX, shows a long thin sickle-shaped flagellate, and Fig. 78 a group of young dividing forms. The contents of the hind intestine contained many active flagellates, and some of these are illustrated in Figs. 70 to 88, Plate XX; they were mostly short, round or long flagellates about to divide. The contents of the rectum contained a very large number of highly motile flagellates, some of which are shown in Figs. 80 to 97, Plate XX. It will be noted that there were many small flagellates with short, stout flagella as shown in the figures.

Bug 5. *Dissected 6 days after the original feed on the culture.* No living flagellates were seen in the contents of the midgut of this bug, but the hind intestine was very full of active parasites, particularly the long sickle-shaped forms. The rectal contents contained a few active flagellates. No smears were made of the contents.

Bug 6. *Dissected 8 days after the original feed on the culture.* The midgut was empty, but when teased up in the saline and examined in the fresh condition a few active flagellates were seen; there were also some apparently dead forms. In Figs. 98 to 104, Plate XX, some small flagellates from this smear of the midgut are illustrated. Fig. 101, Plate XX shows a degenerated round parasite, the nucleus has broken up and now only consists of a number of dark staining granules.

The hind intestine contents contained a large number of active flagellates of all shapes and sizes, many of these are illustrated in Figs. 105 to 129, Plate XX. Several of the round or oval parasites are seen, many with only a small tag of the flagellum left attached to the margin. These forms appeared to predominate in the hind intestine of this bug. Fig. 129, Plate XX, illustrates very long sickle-shaped flagellate undergoing division. In the rectal contents there were a very large number of highly motile flagellates, many of the different types of which

are illustrated in Figs. 130 to 166, Plate XX. The long flagellates were particularly plentiful, as well as small forms with or without flagella. Figs. 137 to 151, Plate XX illustrate some of the latter undergoing simple binary division.

Bug 7. The midgut, hindgut and rectum of this bug were cultured.

Bug 8. *Dissected 10 days after feed on the original culture.* The midgut was empty only containing a few air bubbles. When teased up in a drop of saline solution a few active flagellates were seen in the fresh condition. A short flagellate seen in the stained smear is shown in Fig. 167, Plate XXI. The hind intestine contained very little digested blood, when teased up many short flagellates and long sickle-shaped forms were seen actively moving about in the fluid. The rectal contents were very full of active flagellates, many of which are illustrated in Figs. 168 to 205, Plate XXI: these include short forms with or without flagella, short flagellates and long forms undergoing division.

Bug 9. *Dissected 12 days after feed on the original culture.* The contents of the midgut and hind intestine when teased up in the fresh condition did not contain any parasites. The rectum was full of digested blood which contained many active flagellates; when the smear was stained many round forms were seen some of which are illustrated in Figs. 206 to 217, Plate XXI. Fig. 207, Plate XXI, shows a very small round form like those seen in smears from a sore.

Bugs 10, 11 and 12. The midguts, hind intestines and recta of these bugs were cultured.

Bug 13. *Dissected 16 days after the feed on the original culture* The midgut was empty except for some bubbles of air. A few active flagellates mostly small forms were seen in the fresh condition. The hind intestine contents in the fresh condition were literally swarming with flagellates, and many of the forms seen in the stained smear are illustrated in Figs. 218 to 247, Plate XXI. The rectum contained a few active flagellates, three of which are shown in Figs. 246 to 248, Plate XXI.

Bug 14. The midgut, hind intestine and rectum of this bug were cultured.

Bug 15. *Dissected 19 days after feed on culture.* The midgut was empty and no flagellates were seen when it was teased up in saline solution. The hindgut contents contained a large number of



*Herpetomonas tropica.*



active flagellates, some of which are illustrated in Figs. 250 to 260, Plate XXI.

The remaining bugs' alimentary tracts were cultured.

*Adult bug fed on cultures on 2nd August, and re-fed on human blood on 8th August. Dissected 6 days after original feed on the culture and two days after the clean feed of human blood. Midgut full of young growing flagellates forming rosettes and groups, also many long highly motile flagellates. The stained smear showed a very large number of these forms in all stages of growth and division. A few of these are illustrated in Figs. 261 to 289, Plate XXI.*

No further dissections were carried out.

From these observations of the behaviour of the parasite in the alimentary tract of *Cimex hemiptera* we feel justified in drawing the following conclusions:—

1. When an adult bug is fed on cultures of *Herpetomonas tropica* the flagellates pass down to the rectum of the bug where they can be found in the living condition 24 hours after the feed.

2. In microscopic preparations the parasites can be found in the alimentary tract of the bug as late as the nineteenth day after the feed.

3. From microscopic examinations alone it would appear that the parasite disappears from the midgut of the bug, if it is not re-fed after the original feed on the culture.

4. If a bug is re-fed again on clean human blood after a short interval, a large number of round growing flagellates appear in its midgut contents, and these by multiplying produce an intense infection.

Having ascertained these facts, we realised that by examining the contents of the alimentary tracts of infected bugs microscopically, there is little chance of discovering how long the parasite can live in them unless we know where it finally rounds up. Further, as such examinations are extremely tedious and would take a long time to accomplish, we decided to stop further microscopic examinations but instead to culture the various parts of the alimentary tract of bugs fed on cultures on the NNN medium. To carry this out with any hope of success it was necessary to devise a suitable technique in order to obtain cultures free from bacteria. After many failures, it was found that by first dipping a bug in ether for a few seconds and then brushing both surfaces of its body in 80 per cent. alcohol and with a sterile brush, all surface bacteria were removed. It was

however, first necessary to find the length of time the bug could be immersed in 80 per cent carbolic without the parasites being destroyed in its alimentary tract. We found that by placing infected bugs for two minutes in 80 per cent carbolic we could still obtain cultures of the parasites from the various parts of the alimentary tract; it was clear the carbolic during this time does not penetrate to the alimentary tract of the bug and destroy the parasites.

After brushing the bug thoroughly in the carbolic, an operation which only lasts about a minute, the bug is washed in five changes of sterile saline solution in order to remove all traces of carbolic. The bug is now placed on a flamed slide in a drop of sterile saline solution, and the various parts of the alimentary tract are dissected out with sterile needles, isolated and at once transferred to tubes of NNN medium. They can either be lifted up on a platinum needle, or better still sucked up in a sterile glass pipette of suitable bore. This technique has given us reliable results and we can confidently recommend it.

We have in this way cultured the alimentary tracts of bugs fed on cultures of *Herpetomonas tropica*, and have obtained the following results:—

Adult bugs fed on cultures of <i>H. tropica</i> and not re-fed; cultured on varying days after the feed.	Adult bugs fed on cultures and re-fed on clean blood at short intervals.	Nymphs fed on cultures and re-fed on clean blood at short intervals.
Midgut + up to 23 days ..	Midgut + on 34 days ..	Midgut + 31 days.
Hind intestine + up to 23 days ..	Hind intestine + on 44 days ..	Hind intestine + 36 days.
Rectum + up to 23 days ..	Rectum + on 34 days ..	Rectum + 36 days.

Larvæ fed on cultures and re-fed on clean blood at short intervals.  
Midgut on 9th day.

It will be noted from these results that *H. tropica* can live for 23 days in the alimentary tract of starved bugs; for 34 days in the stomach of re-fed adult bugs; 44 days in the hind intestine and 34 days in the rectum. It can live for 31 days in the midgut of a re-fed nymph; 36 days in the hind intestine and rectum; and for at least nine days in the midgut of a bug fed as a larva.

We believe these results by no means represent the extreme limit of time the parasite can live in the alimentary tract of the bug under

varying conditions. We feel sure if these observations are repeated it will be found that the parasite can live for several months. By adopting this technique it would be possible to find the part of the alimentary tract in which the bug can live for long periods. We have found the 'thick tail' of Cornwall on many occasions both in the midgut and hind intestine of bugs fed on cultures, but we have not followed out the further changes undergone by it. In our opinion these results go a long way in supporting the hypothesis that the bug *Cimex hemiptera* is the true invertebrate host of *Herpetomonas tropica* in India.

*Note by the Senior Author.*—Since the above was written, several months ago, I have been able to confirm Mr. Adie's discovery of the intracellular stage of *Herpetomonas damarensi* in the cells of the midgut of *Cimex hemiptera*. By utilising a simple technique I have found that *H. tropica* has a similar intracellular stage in the cells of the midgut of *Cimex hemiptera*. From what I have now seen of this stage, it is quite clear that many of the parasites illustrated in the Plate, XX and XXI accompanying this paper, represent various phases following on this stage. In examining the contents of the alimentary tracts of the bugs, the midgut, hind intestine and rectum were teased up, and there can be very little doubt most of the intracellular forms were in this way liberated.

I hope on another occasion to give a detailed description of the intracellular stage and the development of the parasites resulting from it; and to demonstrate the flagellates in the cells in sections. In any case this discovery of this stage in the extra-corporeal life history of *H. tropica* conclusively proves that *Cimex hemiptera* is the true invertebrate host of the parasite, a conclusion which I arrived at in 1910 in Cambay.

STUDIES ON THE FLAGELLATES OF THE  
GENERA HERPETOMONAS, CRITHIDIA  
AND RHYNCHODOMONAS.

No. 9.

NOTE ON THE BEHAVIOUR OF HERPETOMONAS DONOVANI  
LAVERAN AND MESNIL IN THE BED BUG, CIMEX  
HEMIPTERA FABRICIUS.

BY

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[Received for publication, January 27, 1921.]

It will be remembered that one of us has described the development of the parasite of kala-azar (*Herpetomonas donovani*) in the bed bug, *Cimex hemiptera*, from the unchanged condition as found in the peripheral blood to the flagellate stage, and later to the rounding up of this stage. These experiments were carried out in Madras by feeding bugs, mostly adults, once on the peripheral blood of patients, films of which contained a large number of parasites. It was, however, found that if the bugs containing the flagellates were re-fed on human blood, most of them disappeared, either being destroyed by the blood or by being engulfed by the leucocytes, but in a few of the bugs the parasites could still be

found in small numbers. These few parasites are very difficult to feed especially if they are in a round non motile stage, either in fresh or stained specimens of the contents of the alimentary tract, particularly at that of the hindgut or intestine. It seemed then very necessary to try and find how long the parasite could persist in the alimentary tract of the bug.

As we were not able to feed bugs on kala-azar patients we decided to feed them on cultures of the parasite as described in another paper, and to culture the various parts of the alimentary tract at varying intervals after the infected feed. We realised that, by this method, we would be certain to detect whether any given bug was infected at the time its alimentary tract was cultured. Whereas if the bug were examined microscopically, there would be less chance of finding the parasite. Accordingly, a large number of bugs, in all stages, were fed on cultures, and dissected at intervals. We have obtained the following results:

1. Adult bugs fed on cultures showing actively dividing flagellates, the bugs not re-fed again. Their midguts, hind intestines and recta cultured on the NNN medium.

The midgut of one of these bugs gave a positive result on the 31st day after the infected feed, the hindgut and rectum of another on the 34th day.

2. Bugs fed on cultures showing actively dividing flagellates, the bugs re-fed at intervals on clean human blood. Their midguts, hindguts and recta cultured on the NNN medium.

The midgut of an adult bug gave a positive culture on the 41st day after the infected feed, and with six succeeding feeds of clean human blood. The hindgut of another bug, fed as above, gave a positive culture on the 34th day after the infected feed. A bug fed as a nymph on a culture was dissected and its midgut, hindgut and rectum gave a positive culture on the 34th day after the infected feed.

It will be noted from these results that *Herpetomonas domoestica* can live as long as 41 days in the midgut of *Cimex hemiptera*, even though the bug be fed six times on clean human blood after the one infected feed. Though we are not in a position to say what stage the parasite are in at the time it was cultured, there could be no doubt it was in a living condition and could infect a tube of NNN medium. Although in the majority of the bugs, we were unable to recover the parasite by cultures in this one bug the infection persisted after 41 days. It is very evident that *H. donovani* can live in the midgut of the bug for a long time, and

even in spite of successive feeds of blood. We have little doubt that when these experiments are repeated it will be found that the parasite can survive even longer.

In concluding these studies, we might point out that we have cultured the alimentary tracts of 1,233 bugs, for the most part using three tubes of NNN medium for each bug. These experiments have conclusively proved that the alimentary tract of the bug is in nature absolutely sterile. All the contaminations were the result of bacteria from the bodies of the bugs infecting the saline in which they were dissected. Further, the senior author has dissected many thousands of bugs during the last 15 years, and has never once found bacteria in any part of the alimentary tracts of bugs fed on human blood in the natural way. Bacterial sterility is an important factor in connection with the extra-corporeal development of *H. donovani*, and thus it is the parasite is able to live and multiply in the alimentary tract of the bug. The alimentary tract of *Pulex irritans*, on the other hand, is frequently contaminated with bacteria. It will be readily understood how this is brought about when we remember that its larva feeds on dirt of all kinds.

PRELIMINARY NOTE ON THE DEVELOPMENT  
OF THE LEISHMAN DONOVAN PARASITE  
IN SPLEEN JUICE AND IN THE  
ALIMENTARY TRACT OF  
*CIMEX LECTULARIUS*  
(LIN.)

BY

HELEN A. ADIE.

(Received for publication, March 5, 1921.)

DURING the latter half of 1919 up to the end of 1920 I have had an opportunity of carrying out feeding experiments with bed bugs *Cimex retundatus* (Sig.) and *C. lectularius* (Lin.) in the Kaiser hospital at Shillong.

The feeding material used was spleen juice mixed with the patient's blood. The spleen puncture was made for diagnosing the disease and the cases were not under treatment.

A complete report on the findings is under preparation but, for the interest of other workers, I submit this short note with figures showing developmental forms observed in the spleen juice and their further development in the alimentary tract of *Cimex lectularius* (Lin.).

The cycle as observed by me presents the following series of forms:

I. DEVELOPMENT IN THE HUMAN SPLEEN

In the spleen there are large intra-cellular parasites dividing by multiple fission producing torpedo-shaped merozoites which can be seen leaving heavily infected broken-down cells in large numbers. Plate XXIII, Fig. 2, illustrates a stained specimen. There are also to be found in other spleen cells developing and multiplying within the still smaller merozoites which are to be seen extra-cellularly. Plate XXIII, Fig 1, illustrates this.

It is probably these second generation merozoites which are to be found in leucocytes in the peripheral blood where they grow and develop, and it is undoubtedly these forms which develop in the alimentary tract of bugs fed on spleen juice.

It might be pointed out, in this connection, that my observations seem to prove that injection experiments in animals from flagellate cultures are far less likely to give results than direct spleen juice injections. Any success resulting from injection of flagellate cultures into animals depends entirely on the presence of preflagellate forms. It is therefore clear that the chances of infection are considerably lessened by using cultures instead of spleen juice direct.

## II. THE DEVELOPMENT IN THE STOMACH OF THE BUG.<sup>4</sup>

These small merozoites, which have been observed in the spleen scattered about extra-cellularly, are to be seen in the bug's stomach, eight hours after a feed, collected together in groups. Plate XXIII, Fig. 1.

They grow and multiply by binary fission and later flagellate. The size of the rosettes thus formed depends on the number of parasites collected together in the original group. At varying periods (depending on various factors, such as amount of blood ingested by the bug, temperature conditions, the degree of infection, etc.) flagellates, freed from rosettes, can be seen visiting many parts of the gut wall trying, apparently, to find a suitable passage for entry into cells. Eventually, their object is attained, and they disappear from view. They remind me of bees visiting flowers in search of honey. This phenomenon was noticed by me early in the investigation and suggested the possibility that the parasites had penetrated the cells.

Later, I was able to observe flagellates boring and burrowing into cells by their posterior ends. Some substance drawn from the host cell appears to be sucked into the parasite, expanding the ectoplasm and forming a capsule which completely surrounds the flagellate but does not obscure it.

Through the transparent capsule the flagellate can be clearly seen to curl and twist and finally to roll up with the flagellum folded round it. The capsule at this stage has become spherical; protrusions are thrust out, caused by the waving of the enclosed folded flagellum. It is difficult to see further development in the fresh specimen, as the parasite burrows down the cell, and one has to focus down in order to follow the rounding-up process.

However, in encapsulated parasites pressed out of the cell, in an accident of dissection still further changes can be seen to take place. In encapsulated forms freed from the cell early in development the parasite, after a time, degenerates. It can be seen to curl and uncurl repeatedly as the flagellum is extended and withdrawn (*see* the stained specimen Plate XXII, Fig. F1). The capsule becomes swollen and the body finally becomes an extraordinary, writhing, wriggling mass of coils. It grows to a colossal size and finally disintegrates. Probably this is a variety of one of Col. Cornwall's 'Thick tails.'

If, on the other hand, the rounding-up stage has been fully attained intra-cellularly and the parasite is then pressed out of the cell, nuclear changes can be seen to take place, resulting in the development of new flagellates. In fully developed spheres the flagellates can be clearly seen writhing and wriggling over each other within the capsule which, at this stage, spins round rather like a top nearing the end of its gyration. Flagella are seen to be extruded and the sphere bursts setting free the enclosed flagellates.

At this point one of two courses is taken by the flagellates. In the case of a heavily infected cell in which all the available nutriment has been exhausted the flagellates set free from the sphere are seen to penetrate other cells and go through the same developmental changes as are described above.

On the other hand, in less heavily infected cells, I have observed the occurrence of syngamy which I shall describe presently.

Some of the flagellates have a single macro-nucleus, others have two. In the heavily infected cell the size of the spheres and the number of flagellates varies according to the available space permitting development. A large sphere may contain as many as 16 and a small sphere as few as 4 or 5 flagellates. In fresh preparations empty sheaths can sometimes be seen.

Plate XXII Figs. F1, F2, F3, F6, F8, F10, F11, F12, show the sphere burst and escape of flagellates, while Plate XXII, Figs. D1, D5, D23, D24, D16, illustrate the double macro-nucleus.

### III. APPEARANCES SUGGESTIVE OF SYNGAMY.

Conjugation between free flagellates has been observed to take place inside cells. Such forms are seen to approach one another; the chromatin becomes scattered; cytoplasmic fusion takes place followed by nuclear fusion. Some of the conjugation forms might

be taken for dividing forms if they had not been seen to approach and fuse.

Plate XXII, Figs. A2, A4, A10, A11, I2, I4, show early conjugation forms.

Two flagellates have been observed to approach and fuse end on. A capsule forms enclosing them taking the contours of the flagellum of one parasite and twisting with it but spreading widely round the flagellum of the other which can be seen waving independently inside the expanded end.

After cytoplasmic fusion of the two forms nuclear changes occur. Flagellates of a type hitherto undescribed are later developed which can be seen to leave the cyst. They are long and slender, sharply pointed and very granular.

Plate XXII, Figs. B1, B2, E2 and E3 show these cystic forms. Later forms are figured in E3, F9, F4 and F7. Freed flagellates are figured in G1, G2, G3. On escaping from the cyst, these freed flagellates coil and uncoil, straighten out and again coil. Their mode of progression on first escaping is rather like that of halteridium sporozoites in the *Lynchia maura*.

Such forms are seen in stained specimens figured in Plate XXII, Figs. G4, G5, G12, sickle-shaped, G6, G7, G8 annular forms. Many of the latter link up later, and are seen in couples and groups as in Plate XXII, Figs. G10, G11, G12.

Ultimately they withdraw the flagellum. (Plate XXII, Fig. G9) and become spherical as in Fig. G13.

No flagellates have been observed in the salivary glands. Possibly a raised temperature is necessary for an invasion of the salivary glands, if it occurs.

#### IV. REMARKS.

From observations made, it appears that the vegetative intra-cellular and propagative phases of Leishman Donovan parasites react to stimuli and environment.

(1) Rheo-taxis effects brought about by the nutriment absorbed by the parasite;

(2) Thermo-taxis effects of temperature with regard to increased vitality.

(3) Chemo-taxis for selection of suitable host cells and later sexual attraction. The stimuli are inter-dependent. It has been observed that if the temperature, favourable to the highest pitch of vital activity, is

exceeded, the Leishman Donovan parasite dies. Perhaps this may explain why kala-azar flourishes in Assam, Madras, the Mediterranean and other regions, where the optimum temperature is not exceeded for prolonged periods.

In the Punjab, the disease would be unlikely to gain ground for the reason that the optimum temperature is exceeded for prolonged periods during both summer and winter. As to a possible explanation of an intra-cellular stage and syngamy observed in spleen juice feeds, it is easy to understand how, in an abnormally heavy infection, vegetative multiplication would soon become arrested owing to the rapid falling off of the food supply. Exhaustion of nutriment in the medium would induce parasites to attack the cells in search of more nutriment. Further multiplication increases the infection completely destroying the cells in many instances.

It is at this stage that I have seen syngamy resorted to. In my opinion it may be due to lack of nutriment causing divergencies in the different strains, and in order to level up such divergencies syngamy is resorted to. Of course, there is the possibility that it may be due merely to the fact that there are no fresh cells left to attack.

It need not, therefore, be concluded that an intra-cellular stage and syngamy is the normal method of reproduction. It must be remembered that in my spleen juice experiments, the degree of infection is abnormally heavy. An intra-cellular stage and syngamy may rarely or never occur in nature as the degree of infection from peripheral blood feeds is much lighter. Divergencies in different strains would not occur and syngamy would be unnecessary. So that an intra-cellular stage and syngamy may not be related in any way to the normal method of reproduction in the invertebrate host. Other figures shown in Plate XXII suggest a possible erithridial stage succeeding the intra-cellular multiplication. Further observations are necessary to decide this point.

Whilst making my observations in Shillong I had no opportunity of seeing Professor Minchin and Thomson's original papers on the development of *Trypansoma lewisi* in the rat flea. The first intra-cellular form pressed out of a cell seen by me in the early summer of 1919 resembled one of the figures in Minchin's book, 'An introduction to the study of protozoa' to which I referred and found a statement to the effect that an account of some of their investigations had not been published. This edition being a new one (1917) published after the death of Professor Minchin I concluded nothing was yet published. In 1921, on enquiry at Gower's, I discovered

the original papers, and on seeing the beautiful illustrations, I was at once convinced that the first part of the intra-cellular stage in the bed bug is exactly similar to *Trypanosoma lewisi* in the rat-flea. I pointed this out to Major Patton who, later, confirmed my discovery of an intra-cellular stage in the bug.

On comparing my Plate XXII and a few of my slides prepared in Shillong I saw that many of the forms that had puzzled me resemble Minchin and Thomson's crithidial forms. A glance at Plate XXII will suggest the probability that the final stages of the extra-corporeal cycle in the bug may prove to be the same as in the case of *Trypanosoma lewisi* in the flea.

Major Patton is now making further observations in Coonoor and will probably soon complete the cycle.

The figures in Plate XXII are mostly from a striking success obtained in a bug that died six days after feeding on heavily infected spleen juice showing many small extra-cellular forms. The bug was found dead on the sixth morning and removed from the cool incubator. It was placed in saline and kept in my laboratory at a temperature of about 27°C for 24 hours before dissection, it had probably been dead about 36 hours. The parasites were exceedingly active and as will be seen are of a different type to culture flagellates and the forms figured in Major Patton's papers in the 'Scientific Memoirs.'

I have observed similar though less striking results in other dead bugs, but have also seen many of these new forms in chloroformed bugs.

I am endeavouring to confirm my results by feeding bugs on peripheral blood under conditions as closely approximated to natural conditions as can be obtained in a laboratory. I have very often obtained development of forms such as is seen in culture, in bugs fed on spleen juice; although this may also occur in bugs fed from peripheral blood it is no indication of the bug being infective.

EXPLANATION OF PLATE XXII.

A variety of forms iptra-cellularly developed in *Cimex lectularius* (Lin.)  
fed on kala-azar spleen juice in Shillong.

EXPLANATION OF PLATE XII.

A variety of forms intra-cellularly developed in *Cinnamomum* (Pin).  
fed on kals-axar spleen juice in Shilling.



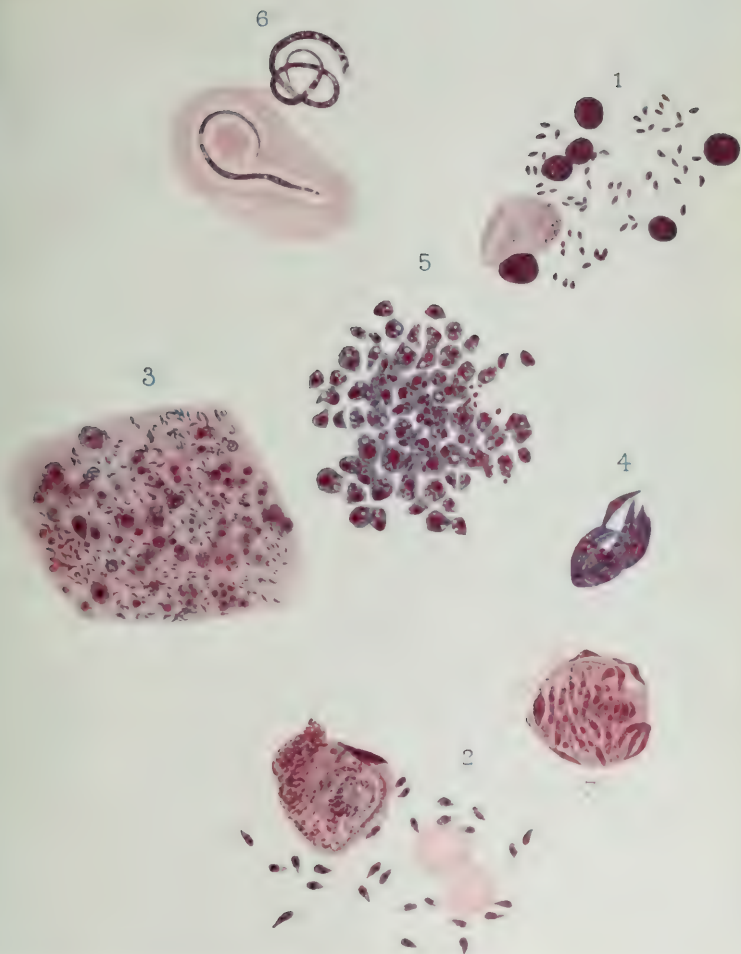


### EXPLANATION OF PLATE XXIII

- Fig 1. Small merozoites from kala-azar spleen juice smear
- .. 2. Larger forms.
- .. 3. Portions of the gut of *Camea lectularius* with a heavy intra cellular infection. The cells of the gut are almost completely destroyed. Most of the flagellates have been freed from the spheres.
- .. 4. A more highly magnified group freed from a sphere in the same specimen.
- .. 5. A cluster of round forms in the bug dividing by multiple fission.
- .. 6. Nematode found in the neighbourhood of the rectum in kala-azar spleen juice-fed bugs.
- .. 7. Bursting spheres pressed out of cells

# EXPLANATION OF PLATE XXIII

- |      |    |                                                                                                                                                                                                |
|------|----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Fig. | 1. | Small merozoites from kalamazoo spleen juice smear                                                                                                                                             |
| "    | 2. | Large form                                                                                                                                                                                     |
| "    | 3. | Portions of the gut of <i>C. wileyi</i> with a heavy intra-cellular infection. The cells of the gut are almost completely destroyed. Most of the flagellates have been freed from the spheres. |
| "    | 4. | A more highly magnified group freed from a sphere in the same specimen.                                                                                                                        |
| "    | 5. | A cluster of round forms in the bag dividing by multiple fission                                                                                                                               |
| "    | 6. | Zenarode found in the neighborhood of the rectum in kalamazoo spleen juice-fed pigs                                                                                                            |
| "    | 7. | Bursting spheres pressed out of cells                                                                                                                                                          |



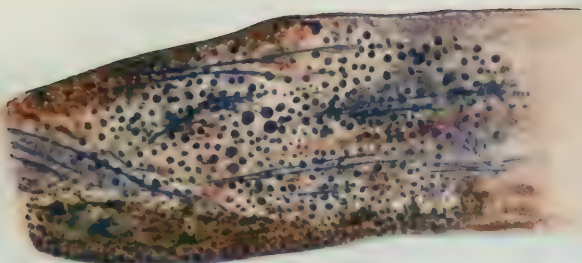
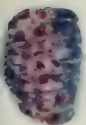


EXPLANATION OF PLATE XXIV.

- Fig. 1. Heavily infected gut of *Cimex lectularius* (Lin). The cells of the gut have been completely destroyed. Some of the darkly stained spheres are nuclei of degenerated cells.
- „ 2. One of the spheres more highly magnified

EXPLANATION OF PLATE XXIV.

- Fig. 1. Heavily infected gut of *Amoeba* (Lin). The cells of the gut have been completely destroyed. Some of the darkly stained spheres are nuclei of degenerated cells.
2. One of the spheres more highly magnified.



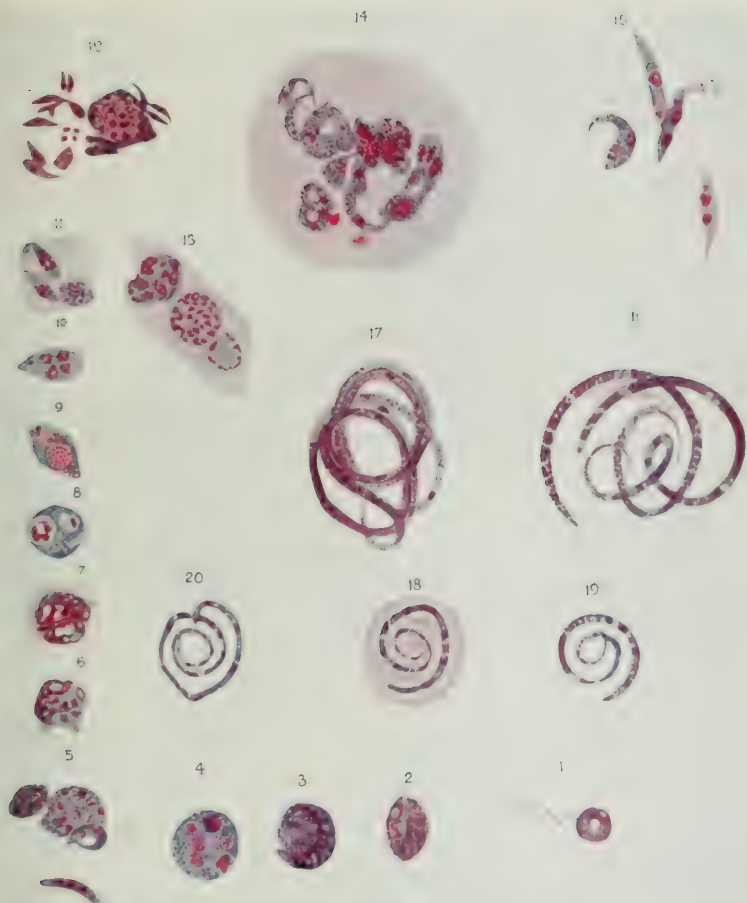


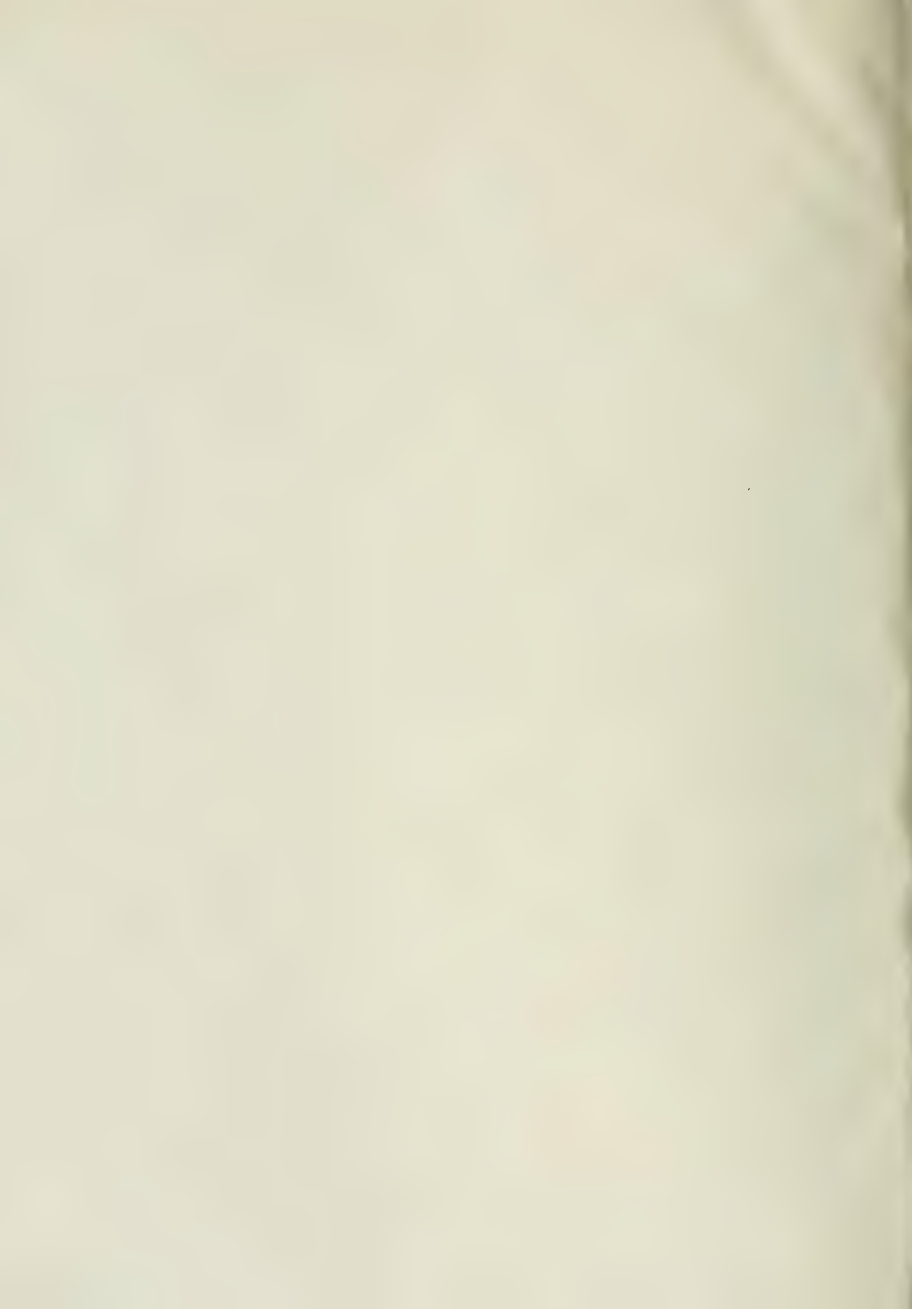
#### EXPLANATION OF PLATE XXV

- Figs 1 to 15 Various stages of intra-cellular forms  
.. 16 to 20 Nematode like structures found in *Cimex lectularius* fed  
on kala-azar infected spleen mice. These structures  
were found in 5 or 6 *Cimex lectularius* bugs fed on  
different patients in whose blood no filaria were  
found after a careful examination of films taken day  
and night.

# EXPLANATION OF PLATE XXX

Fig. 1 to 15 Various stages of intra-collular form  
 .. 16 to 20 Zonitoid-like structures found in *C. maculosa* but  
 on Kabaner infected sheep liver. These structures  
 were found in 5 or 6 *C. maculosa* but on  
 different patients in whose blood no filariae were  
 found after a careful examination of films taken day  
 and night





# BACTERIOLOGICAL AND LABORATORY TECHNIQUE.

## Section III.

BY

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[Received for publication, 11th March 1924.]

### INTRODUCTION.

IN the preparation of this section I must acknowledge my special indebtedness to Kolmer's work 'Infection Immunity and Specific Therapy.'

### B8 BLOOD SERUM REACTIONS<sup>1</sup>.

**Notes.**—<sup>1</sup>Used for diagnosis of disease or for identification of organisms with test serum or test antigen as the case may be. The procedure of standardization of serum and of antigen also involves the use of these reactions.

### B8·1 AGGLUTINATION REACTION.

**B8·11: B8·111 ANTIGEN<sup>1</sup>.**—(1) Use as antigen (**A3·211**) a suspension of a 24-hr. agar culture of the appropriate organism standardized by turbidity (**S7 12**) and diluted to contain 1 mgm. dried bacterial substance per c.c.

**Notes.**—<sup>2</sup>In this description a suspension of living bacteria used. Other antigen suspensions which may be used are suspensions in salt solution or 24-hr. bouillon cultures in which the organisms are killed by the addition of an equal volume of 5 per cent formalized 0·85 S.S.S. (**A3·219**), or by autoclaving (**S9·31**) at 121° C. for 2 min. or longer and the suspension sterilized by heat, for a few minutes. If the standardization of the antigen is the object of the test the antigen is given to the organism (**S9·3**). When a serum is the object of test a known organism will furnish the antigen (**S12·2**).

strengths are very commonly used. If the titre of the serum is to be followed out in the finest detail, less concentrated suspensions are said to give better results.

**B8-112 TEST.**<sup>1</sup> (1) Have in readiness a rack<sup>2</sup> with 13 small<sup>3</sup> T. T., a porcelain slab with 12 depressions, a set of watch glasses, Wright capillary pipettes fitted with teats, and a tin of boiling water with Bunsen flame beneath. (2) Mix equal vol. of 0.85 S.S.S. and antigen suspension in a watch glass. (3) Add the mixture to No. 13 T.T. as 'salt control.' (4) Mark a capillary pipette with a green pencil at a point about 4 to 5 in. up the stem as unit vol. (5) Set out unit vol.<sup>4</sup> of 0.85 S.S.S. in each of the depressions of the porcelain slab. (6) Take up unit vol. of test serum and add to the salt sol. in the 1st depression. (7) Mix, and remove<sup>5</sup> one unit vol. of the mixture. (8) Add the removed vol. to the salt sol. in the 2nd depression.<sup>6</sup> (9) Mix, and remove unit vol. of this mixture to the salt sol. in the 3rd depression. (10) Continue the process to the 12th depression at which the removed vol. is simply rejected.<sup>7</sup> (11) Wash out the capillary pipette well with boiling water. (12) Add one unit vol. of antigen suspension to each of the serum dilutions, beginning with the twelfth and passing up to the first. (13) Mix at the time of each addition. (14) Wash out the capillary pipette well with boiling water. (15) Take up samples of the serum-antigen mixtures in due order, beginning with that containing the greatest dilution of serum and continuing to that with the least. (16) Place each sample as it is taken up in its appropriate T.T. (17) Examine each T.T. with the hand lens and record as 'immediate' the agglutination which has already taken place, with its degree and the dilution of serum. (18) Keep the T.T. 2 hr.<sup>8</sup> in a water bath at 50 to 55C.<sup>9</sup> (19) Keep 15 to 20 min. at R.T. to allow sedimentation of flocculi to take place. (20) Examine and record the degree of agglutination which has taken place for each dilution of serum in terms of turbidity as compared with that of the 'salt control.' (21) Invert<sup>10</sup> the T.T. (22) Leave in the inverted position 20 or 30 min. (23) Restore to their original position. (24) Examine and record the degree of agglutination which has taken place for each dilution of serum in terms of size of flocculi. (25) Keep 18 hr. at R.T. (26) Record the degree of agglutination which has taken place in terms of turbidity and flocculation for each dilution of serum.

**Notes.**—<sup>1</sup> The general adoption of prophylactic inoculation has curtailed the usefulness of the test as a means of diagnosis. The test, in inoculated individuals, must show, on repetition, values fluctuating in accordance with the progress of the disease, for any given organism before that organism can be incriminated as the causal agent in the disease. It is well that samples of blood which are to be used for the agglutination test should be collected aseptically, as the clot can then also be used for blood culture. <sup>2</sup> In

some respects what is better than a rack, as being easier to manipulate, a tube covered at one end with plasticine in which the T.T. are imbedded. <sup>3</sup> The tube is covered to the internal diameter. The less the internal diameter of the tube, the more accurate the agglutination. <sup>4</sup> Instead of using a capillary pipette and a graduated unit vol. pipette, measuring cubic centimetres and fractions of a cubic centimetre may be used. The use of such a pipette is necessitated when the 'titre' of a serum is defined as that quantity of serum which just suffices in 1 hr. at 37°C to cause the appearance of flocculi visible to the naked eye in 1 c.c. of a suspension of 2 mgm. of an 18 hr. culture in 0.85 S.S.S. Drops furnished from a pipette with a test may replace unit vol. <sup>5</sup> In the process of taking up a unit vol. of serum or serum dilution, and in mixing, avoid carrying fluid above the mark, as that may leave there a quantum of serum which may be carried on to a much lower dilution and thus vitiate the results. <sup>6</sup> When the test is for the diagnosis of infection the dilution of the test serum will seldom have to be taken as far as the 12th tube. In the case of a high titre serum the dilution may sometimes have to be carried further to obtain a zero reading. <sup>7</sup> There are thus left at this stage in each depression unit vol. of 1.2, 4, 8, 16, 32, 64, 128, 256, 512, 1,024, 2,048 and 4,096 dilution of serum. <sup>8</sup> Some organisms require longer time. Typhoid group organisms are agglutinated in 2 hr., dysentery organisms in 4 to 6 hr., and meningococci in 24 hr., <sup>9</sup> This temperature is an optimum one for agglutination. An incubator temperature of 37°C however is quite satisfactory in most cases. <sup>10</sup> The inversion is possible if the T.T. are imbedded in plasticine. With T.T. of the dimensions given the column of fluid contained does not move from its position. Readings may be made without inversion. The object of the inversion is to differentiate between simple sedimentation and flocculi. It also serves to separate out the flocculi, which allows of their size being determined.

**B8-12: B8 121 ANTIGEN<sup>1</sup>.** (1) Use as antigen (A3-211) a suspension of a 24-hr. agar culture of the organism.

**Notes.**—Where an organism such as *B. pestis* is spontaneously agglutinable the suspension should be made with 1-1000 salt sol. instead of 0.85 S.S.S. In the case of *B. tuberculosis* the felted growth may be brought into homogeneous suspension by prolonged trituration in 1-1000 salt sol. When highly dangerous organisms are being dealt with special methods (A3-221) are employed to produce the antigen suspension.

**B8-122 TEST.**—(1) Make a series of dilutions of test serum with 0.85 S.S.S. (2) Set out a series of unit vol. of each dilution of antigen and a unit vol. of 0.85 S.S.S. for control. (3) Wash out thoroughly the pipette used. (4) Fill into the pipette as many unit vol. of the antigen suspension as there are unit vol. set out on the slides. (5) Eject one vol. of the antigen suspension into each unit vol. on the slides beginning with the vol. of 0.85 S.S.S. (6) Mix serum and antigen before proceeding to eject the next vol. of suspension. (7) Wash out thoroughly the pipette used. (8) Aspirate into the pipette, beginning with the control, a sufficient sample of each mixture. (9) Keep the mixtures separate in the stem of the capillary pipette by means of air bubbles and

(10) Seal the orifice of the pipette in the flame. (11) Set the pipette upright. (12) Read the results<sup>1</sup> after 24 hr.

**Notes.**—<sup>1</sup>The end point taken may be either one of complete sedimentation with clearing of the S. N. F. or the highest dilution of serum giving rise to macroscopically evident flocculation. A hand lens examination is useful to supplement the naked eye examination. Simple sedimentation may be differentiated from sedimentation of flocculi by inverting the pipette, when, if the sedimentation has been due to gravitation alone, the deposit will resolve itself into a shower of impalpably fine articles whereas if the sedimentation is of flocculi the shower will consist of comparatively large agglomerated masses of bacteria.

**B8-13: B8-131 ANTIGEN.**—(1) Use ordinary veal peptone bouillon<sup>1</sup> as medium in quantities of 1 litre contained in a 1½ litre flask. (2) Sterilize 15 min. only at 115C. (3) Test sterility by incubation for 72 hr. (4) Sow from a 24-hr. bouillon culture<sup>2</sup> of the testing organism. (5) Incubate 24 hr. (6) Shake thoroughly to mix. (7) Add 0·1 per cent formalin. (8) Shake to mix. (9) Keep in the ice chest at about 2C. (10) Shake thoroughly to mix at intervals throughout the day and for 4 days thereafter. (11) Replace<sup>3</sup> in the ice chest.

**Notes.**—<sup>1</sup>The bouillon is standardized to a phenolphthalein indicator, and ⅓rd of that amount of sod. hydroxide which would render it neutral to phenolphthalein is added before the final boiling and filtration. <sup>2</sup>The culture used for sowing should be one which has been subcultured daily in bouillon for 2 weeks or longer. This continued subculture has the effect of increasing its agglutinability and diminishing any tendency to spontaneous agglutination. <sup>3</sup>After 4d. the cultures will be found to be absolutely sterilized. The culture, if it is not homogeneous, may be filtered sterily through cotton wool.

**B8-132 ANTIGEN STANDARDIZATION OPACITY.**—(1) Set up two rows of 10 small T.T. of the same internal diameter containing in the first row serial dilutions of 2, 3, 4, 5, 6, 7, 8, 10, 12 and 16 in 20 of a 'standardized' antigen suspension, and in the second row the same dilutions of the antigen to be standardized. (2) Make a comparison of the opacities of the T.T. in the two rows and find the degree of opacity of the antigen to be standardized in terms of the 'standardized' antigen. (3) Dilute the antigen to be standardized to equality with the 'standardized' antigen with 0·1 per cent formalinized 0·85 S.S.S.

**Notes.**—<sup>1</sup>Dysentery cultures should, after dilution to 'standard' opacity, be left to stand in the ice chest for 6 weeks before being standardized for agglutinability.

#### **B8-133 ANTIGEN STANDARDIZATION AGGLUTINABILITY.**

(1) Set up two rows of 10 small T.T. (2) Prepare a dilution of 'standard' serum to contain 4 to 8 'standard' agglutinin units. (3) Dilute this again with 0·85 S.S.S. 2, 3, 4, 5, 6, 7, 8, 10, 12 and 16 in 20,

(4) Mix 2 vol. of each of these dilutions with 3 vol. of 'standardized' antigen suspension in the T.T. of the front row. (5) Do likewise for the test suspension<sup>1</sup> in the T.T. of the second row. (6) Keep the T.T. 2 hr.<sup>2</sup> at 50 to 55C. (7) Keep 20 min. at R.T. (8) Select the T.T. in each row showing 'standard' agglutination.<sup>3</sup> (9) Express the degree of agglutinability of the test suspension in terms of the 'standardized' antigen and give the former the appropriate factor<sup>4</sup> which will make it also a standard antigen in agglutination tests. (10) Use this new factor in agglutination tests with the newly standardized antigen to determine the number of 'standard' agglutinin units<sup>5</sup> contained in 1 c.c. of a test serum.

**Notes.**—<sup>1</sup>Approximately of the same opacity as the 'standardized' antigen suspension. <sup>2</sup>Dysentery 4 to 5 hr. <sup>3</sup>The highest dilution of serum in which marked agglutination without sedimentation can be detected by naked eye. <sup>4</sup>Multiply the factor given with the 'standardized' antigen suspension by the comparative degree of agglutinability of the antigen to be standardized. Thus, if the former is twice as agglutinable as the latter, multiply the factor by 2, if half as agglutinable, multiply by  $\frac{1}{2}$ . <sup>5</sup>The 'standard' agglutinin unit (Oxford standard) is that amount of agglutinating serum which, when made up to 1 c.c. (or 2 unit vol.) with 0.85 S.S.S., causes standard agglutination on being mixed with 1.5 c.c. (or 3 unit vol.) of a standard agglutinable culture, after 1 hr. at 50 to 55C.

**B8.134 TEST.** (1) Set up a series of 4 small T.T. containing 2 unit vol. of test serum in the dilutions<sup>1</sup> 1-10, 20, 50, 100. (2) Set up a control T.T. containing 2 unit vol. 0.85 S.S.S. (3) Add to each T.T. 3 unit vol. antigen suspension.<sup>2</sup> (4) Shake each T.T. carefully to mix. (5) Keep 2 hr. in a water bath at 50 to 55C. (6) Keep 15 min. at R.T. (7) Examine and record the dilution of test serum giving 'standard' agglutination.<sup>3</sup> (8) Calculate<sup>4</sup> the number of 'standard' agglutinin units per c.c. in the test serum.

**Notes.**—<sup>1</sup>Higher dilutions may be necessary. <sup>2</sup>The dilutions of antigen suspension are 1-25, 50, 125 and 250. <sup>3</sup>The signs and factors used by the Oxford laboratory for indicating degree of agglutination are:—Total agglutination T—complete clarification of antigen-serum mixture; standard agglutination S—flocculation apparent to naked eye inspection; trace tr.—flocculation barely apparent to naked eye inspection. Grades intermediate between these are given by the symbols T-, S-, tr., tr., tr.+, tr.-. Standardization is effected by taking standard agglutination as the end point. Any of the other points may be taken as end point, and reduction to standard agglutination effected through factors. These factors are:—T, 1.47; T-, 1.29; S-, 1.3; S, 1; tr., 0.88; tr., 0.77; tr., 0.68; tr., 0.60; tr., 0.50; tr., 0.46; i.e., if tr. + agglutination is given by 1-50 serum it would be equivalent to 100 given by 1.50,  $\times 1.0771$  in 1-38.5 serum. <sup>4</sup>Divide the number of 'standard' agglutinin units giving 'standard' agglutination with the standard agglutinin antigen by the

the factor attached to the antigen, *e.g.*, if the dilution is 1:250 and the factor 2·5, the number of agglutinin units per c.c. of test serum will be  $250 \div 2 \cdot 5 = 100$ .

**B8·14 SLIDE METHOD.** (1) Use a heavy suspension of the test bacterium and a dilution of the anti-serum containing 40 times as much serum as the titre dilution.<sup>1</sup> (2) Mix on a slide equal vol. bacterial suspension and serum dilution. (3) Use appropriate controls. (4) Note the occurrence within 2 min. of macroscopic agglutination.<sup>2</sup>

**Notes.**—<sup>1</sup>The use of the titre dilution itself would require too long for the action of the serum to be manifested. Too low a dilution of the serum should not be used if paradoxical group agglutinations are to be excluded. If the titre dilution were 1:2000 then the dilution to use would be 1:50. <sup>2</sup>If the mixture is kept in movement, as by rocking the slide, agglutination will be accelerated; this is the principle employed in the agglutinator. The method is useful as a preliminary means of selecting colonies from a plate for further test. It is also used with a known bacterial antigen for the identification of a test serum.

**B8·15 MICROSCOPIC : B8·151 HANGING DROP METHOD.**—

(1) Use a capillary pipette with a unit vol. mark on the capillary stem about 3 cm. from the extremity. (2) Take up one by one unit vol. of 0·85 S.S.S. (3) Eject each vol. of 0·85 S.S.S. as it is taken up<sup>1</sup> into a watch-glass placed ready for the making of the dilution. (4) Take up one unit vol. of test serum and add it to the 0·85 S.S.S. in the watch-glass. (5) Mix well. (6) Make from the dilution of test serum so made, and in similar fashion (*cf.* **B8·112**) further dilutions. (7) Mix on microscope slides equal volumes of antigen suspension<sup>2</sup> and test serum dilution. (8) Cover the mixtures with cover-glasses. (9) Seal the edges of the cover-glasses with vaselin. (10) Set up normal serum and salt controls, in which normal serum dilution and 0·85 S.S.S. take the place of the test serum dilution. (11) Examine the preparations immediately, after 15 min., and after 60 min. with a dry 1·6th. in objective.

**Notes.**—<sup>1</sup>Do not attempt to carry the successive volumes up the stem as this wets the glass to a greater extent than is necessary and results in considerable loss of fluid. <sup>2</sup>Having 24-hr. bouillon culture or a suspension of a 24-hr. agar culture in 0·85 S.S.S.

**B8·152 CELL METHOD.** (1) Use small glass cells, ground flat top and bottom, so that they can be piled in series. (2) Carry out the dilutions of the serum in these cells (*cf.* **B8·112**), and add an equal quantity of antigen suspension to each serum dilution. (3) Stack the cells one on top of the other, with a glass cover to the topmost one. (4) Keep 2 hr. at 37°C. (5) Examine each of the mixtures for agglutination macroscopically and under a low power of the microscope.

**B8-153 THREAD REACTION.** (1) Use a nutrient bouillon containing 1 per cent sod. citrate. (2) Sow with the appropriate organism. (3) Mix with sterile precautions test blood 1 culture bouillon, already sown, 15. (4) Incubate the mixture 5 hr. (5) Examine the clear S.N.F. microscopically for chain or thread<sup>1</sup> formation of the organism used.

**Notes.**—<sup>1</sup>Organisms tend to grow in thread forms in the presence of specific anti-serum.

**B8-16.**—(1) Inject a G.P. or other laboratory animal subcutaneously every second day and 7 times in all, with 2 c.c. 48-hr. bouillon culture of the test organism. (2) Test the serum of the inoculated animal against known organisms for agglutinating effect. (3) Establish the identity of the test organism from the result.

**B8-17 INAGGLUTINABLE STRAINS: B8-171.**—(1) Make a suspension of the organism of strength 0.5 mgm. per c.c. of 0.85 S.S.S. (2) Add 0.1 c.c. N-1 hydrochloric acid to 20 c.c. suspension. (3) Keep 5 min. at 100°C. (4) Cool rapidly. (5) Neutralize with 0.1 c.c. N-4 sod. hydroxide. (6) Use<sup>1</sup> in test.

**Notes.**—<sup>1</sup>Applied to gonococcus and pneumococcus.

**B8-172.** (1) Subculture the inagglutinable strain for several days. (2) Re-test agglutinability.

**B8-18 MENINGOCOCCUS: B8-181.** (1) Use for the antigen suspension in an agglutination test, growths of not more than 14 hr. incubation.

**B8-182.**—(1) Make a suspension from a 24-hr. glucose ascitic agar culture. (2) Shake thoroughly. (3) Allow to stand 4 hr. (4) Use the S.N.F. as antigen suspension<sup>1</sup> for agglutination test.

**Notes.**—<sup>1</sup>Will not deposit the meningococci for 2 or 3d.

**B8-183.**—(1) Make the suspension in 0.85 S.S.S. of a 24 hr. legume or other agar culture of the test organism. (2) Keep<sup>1</sup> 30 min. at 65°C. (3) Set up a series of 5 small T.T. each containing 0.2 c.c. antigen suspension. (4) Add to each T.T. in order 1 c.c. 0.85 S.S.S., 1 c.c. 1-100, 200, 400 anti-serum,<sup>2</sup> and 1 c.c. 1-50 normal serum. (5) Keep 24 hr. at 55°C. (6) Read the results. (7) Keep 18 to 24 hr. in the ice chest. (8) Read the results again.

**Notes.**—<sup>1</sup>The suspension thus treated may be left for some 48 hours at 55°C. and the S.N.F. only used in test. <sup>2</sup>The antiserum used may be a precipitating type serum for the identification of the type of meningococcus.

**B8·19 STREPTOCOCCUS : B8·191.** (1) Wash the organism several times with D.W. (2) Re-suspend in D.W. for use in agglutination test.

**B8·192.**—(1) Prepare a medium: trypt-bouillon (**M4·711**) 2. serum or hydrocele fluid, heated 30 min. at 60°C. 1. (2) Sow. (3) Incubate the culture in the sloping position. (4) Shake<sup>1</sup> up the culture. (5) Dilute to suitable strength for use in test.

**Notes.**—<sup>1</sup> The streptococci grow in very short chains and a uniform suspension is obtained on shaking.

**B8·193.**—(1) Grow the streptococcus in a bouillon of pH 7·4 in which the sod. chloride is replaced by a balanced phosphate mixture. (2) Remove the organism from the culture by centrifugalization. (3) Wash twice with bouillon prepared as above. (4) Re-suspend in this bouillon for use in test.

**B8·2 AGGLUTINATION REACTION : B8·21 PNEUMOCOCCUS INFECTION TYPE.** (1) Wash test pneumonic sputum with sterile 0·85 S.S.S. (2) Inject 1 c.c. intraperitoneally in a mouse. (3) Kill the mouse after 6 hr. (4) Wash out the peritoneum with 8 c.c. 0·85 S.S.S. (5) Centrifuge the turbid fluid obtained at low speed to throw down tissue cells. (6) Transfer the S.N.F. to a second centrifuge tube. (7) Centrifuge at high speed to throw down pneumococci. (8) Use the deposit as antigen suspension in agglutination test in mixture with dilution of type sera. (9) Determine the type from the agglutination results.

**B8·22 B. TUBERCULOSIS : B8·221.** (1) Keep 1 grm. well-dried bacilli in 0·5 per cent sod. hydroxide 8d. at 37°C. (2) Neutralize the opalescent fluid with acetic acid. (3) Use as antigen suspension in agglutination test.

**B8·222.**—(1) Prepare a standard suspension in 0·85 S.S.S. of killed *B. tuberculosis*. (2) Centrifugalize the standard suspension till the deposit obtained is of constant vol. (3) Use the S.N.F. which will itself be standard. as antigen suspension for agglutination test. (4) Set up a series of small T.T. containing mixtures of suspension and varying dilutions of test serum. (5) Keep 5 hr. at 37°C. (6) Examine.

**B8·223.**—(1) Sow from a culture of human *B. tuberculosis* on potato into 50 c.c. 6 per cent glycerin bouillon in half filled flasks. (2) Shake up the bouillon culture daily. (3) Subculture once monthly. (4) Use these cultures as antigen suspension for agglutination test after 35d. incubation. (5) Dilute with 0·85 S.S.S. to render the

suspension sufficiently agglutinable. (6) Keep for use either in the ice chest or after addition of formalin. (7) Use within 2 weeks of preparation. (8) Set up test serum antigen, and control mixtures. (9) Keep 3 hr. at 50 to 55°C. (10) Examine.

**B8:23 B. MALLEI : B8:231.** (1) Use a suitable agglutinable strain of *B. mallei*. (2) Maintain its agglutinability by subpageage through a G.P. at least once every 3 weeks. (3) Grow 60 hr. on 5 per cent glycerin agar 2.9 per cent acid to phenolphthalein. (4) Make a suspension of the growth from the agar slope in 0.5 per cent carbolized 0.85 S.S.S. (5) Keep 2 hr. at 60°C. (6) Shake well to render the suspension homogeneous. (7) Filter through sterile filter paper. (8) Dilute with 0.5 per cent carbolized 0.85 S.S.S. until the suspension is of only faintly cloudy appearance. (9) Set up agglutination mixtures of this antigen suspension and dilutions of the test serum. (10) Keep 2 hr. at 37°C. (11) Examine.

**B8:232.**—(1) Use 48-hr. cultures on glycerin agar. (2) Heat 2 hr. at 60°C. (3) Make a suspension of weak milky appearance with 0.5 per cent carbolized 0.85 S.S.S. (4) Filter the suspension quickly through sterile thin filter paper. (5) Set up the usual test agglutination mixtures of this antigen suspension and dilutions of test serum. (6) Keep 2 hr. at 50 to 55°C. (7) Examine.

**B8:24 B. ANTHRACIS : B8:241 ANTIGEN.** (1) Subculture *B. anthracis* daily on ordinary agar, incubating at 42°C. until a non-sporing form is obtained. (2) Wash off the growth in 0.5 per cent formalinized 0.85 S.S.S. (3) Shake 48 hr. (4) Keep several days at R.T. (5) Test sterility. (6) Shake 24 hr. (7) Keep 18 hr. at R.T. (8) Filter the upper layers of the suspension through filter paper. (9) Dilute with 0.5 per cent formalinized 0.85 S.S.S. to a turbidity corresponding to a suspension of *B. typhosus* of 2000 million per c.c.

**B8:242 TEST.** (1) Set up agglutination mixtures of the antigen suspension and dilutions of the test serum. (2) Keep 2 hr. at 50 to 55°C. (3) Examine.

**B8:25 SP. PALLIDA : B8:251 ANTISERUM.** (1) Grow *S. pallida* 14 to 30d. in the medium:—rabbit serum 1 l.; faintly alkaline bouillon 1; containing a piece of fresh rabbit kidney. (2) Inject rabbits every 7d. intravenously with 1.5 to 3 c.c. culture.

**Notes.**—The human ascitic fluid commonly used is covered by a layer of oil in order to avoid the production of a specific precipitin or of complement-fixing units with human proteins.

**B8·252 TEST.**—(1) Set up a series of small T.T. containing 0·1 up to 0·000001 c.c. anti-serum in each. (2) Add 0·1 c.c. test spirochæte antigen suspension. (3) Bring the final vol. up to 1·5 c.c. (4) Shake gently to mix. (5) Keep 2 hr. at 37C. (6) Read results. (7) Read again after 2 hr. at 37C.

**B8·26 SP. ICTEROHÆMORRHAGIÆ : B8·261 ANTIGEN.**—Use antigen suspensions of living organisms in test, prepared from rich cultures grown 2 to 3 weeks in the medium :—rabbit serum 1 ; 0·9 per cent sterile salt sol. 3.

**B8·262 TEST.**—(1) Set up a series of small T.T. containing 0·1 up to 0·000001 c.c. test serum in each. (2) Add 1 c.c. antigen suspension. (3) Bring the final vol. up to 1·5 c.c. (4) Shake gently to mix. (5) Set up controls. (6) Keep 2 hr. at 37C. (7) Examine the contents of the T.T. for clumping by dark field illumination.

**B8·27 LEPTOSPIRA ICTEROIDES.**—(1) Proceed as in **B8·25** or **B8·26**.

**B8·28 SPOROTRICHOSIS : B8·281.**—(1) Grind up the growth of a 4-week agar culture with 0·85 S.S.S. to make the antigen suspension. (2) Set up test serum, antigen, and control mixtures. (3) Examine the mixtures microscopically after a few min. for agglutination of the spores. (4) Examine the mixtures macroscopically after 6 hr. at R.T.

**B8·282.<sup>1</sup>**—(1) Rub up a large loopful of a 9-week culture of *Sporothrix* in a mortar with 0·85 S.S.S. (2) Filter the suspension through moistened filter paper. (3) Examine microscopically to see that the suspension consists of free spores only and in sufficient number. (4) Use for agglutination test.

**Notes.**—<sup>1</sup>The serum from a patient suffering from sporotrichosis agglutinates the spores in 60 min. in 1:400 or 500 dilution.

**B8·3 AGGLUTINATION REACTION : B8·31 ABSORPTION TEST : B8·311 ANTIGEN.** (1) Make a suspension of a 24-hr. agar culture of the test organism in 0·85 S.S.S. to contain about 5000 million living organisms per c.c. (2) Make a similar suspension of the organism homologous to the anti-serum selected.

**B8·312 TEST OF ANTIGEN.**—(1) Place 1 c.c. undiluted anti-serum<sup>1</sup> into each of 3 clean centrifuge tubes. (2) Add to No. 1 tube 2·9 c.c. 0·85 S.S.S. (3) Add to No. 2 tube 4 c.c. 0·85 S.S.S. and 5 c.c. test antigen suspension. (4) Add to No. 3 tube 4 c.c. 0·85 S.S.S. and 5 c.c. antigen suspension of the organism homologous to the anti-serum. (5) Shake

to mix. (6) Keep 2 hr. at R.T. (7) Keep 1 hr. at 37°C. (8) Centrifuge at high speed. (9) Determine the agglutination titre for the S.N.F. in each of the 3 centrifuge tubes for any desired number of organisms or types of organisms including the test organism and the organism homologous to the anti-serum. (10) Compare the results<sup>2</sup> obtained.

**Notes.** —<sup>1</sup>The anti-serum selected is that which gives by previous trial the highest agglutination with the test organism. <sup>2</sup>No antigen suspension is added to No. 1 tube, but the dilution of serum in this tube is exactly the same as that in Nos. 2 and 3 tubes. The agglutination result shown by the fluid in No. 1 tube affords a means of direct comparison of titre with that shown by the same dilution of serum after absorption in tubes Nos. 2 and 3. <sup>3</sup>If the test organism absorbs all agglutinins primary or secondary (group) to the homologous organism from the selected anti-serum it may be adjudged to be identical with the latter. This result may be further investigated by absorbing another anti-serum with the test organism and with the organism homologous to the originally selected anti-serum, when it should be found that all agglutinin for these two organisms has been removed, but that the agglutinin to the organism homologous to the second anti-serum remains. In absorption tests the anti-serum must show complete absence of agglutinin for the absorbing antigen, otherwise any remaining agglutinin cannot be adjudged to be primary or secondary (group). If therefore in the test residual agglutinin for the absorbing antigen is present further addition of that antigen must be made in order completely to remove agglutinin. An anti-serum cannot be exhausted of primary agglutinins by an organism of a species other than the homologous organism. It is always exhausted by its homologous organism.

**B8:313 TEST OF SERUM.**—(1) Determine the agglutination titre of the test serum to a series of suitable organisms. (2) Absorb the serum with suspensions of these organisms as described in **B8:312**. (3) Determine the extent to which the absorbed serum has been deprived of both primary and secondary (group) agglutinins to each of the organisms used for absorption.

### **B8:32 ABSORPTION TEST MENINGOCOCCUS.<sup>1</sup>**

**Notes.** —<sup>1</sup>The methods given may be considered applicable to any organism which requires classification according to serological type.

**B8:321.**<sup>1</sup>—(1) Make suspensions with 0.85 S.S.S. of 24-hr. growth on legumin tryptic agar which should contain no blood or other body fluid. (2) Keep 30 min. at 65°C. (3) Dilute the suspension to a strength of 4000 million per c.c. (4) Add phenol to 0.5 per cent. (5) Set up 6 centrifuge tubes each containing 2 c.c. 1-25 selected<sup>2</sup> type meningococcus serum. (6) Add to No. 1 tube 2 c.c. suspension of known homologous type meningococcus, to No. 2 tube 2 c.c. suspension of test organism, to No. 3 tube 2 c.c. first heterologous<sup>3</sup> type meningococcus, to No. 4 tube

2 c.c. second heterologous type meningococcus, to No. 5 tube 2 c.c. third<sup>4</sup> heterologous type meningococcus and to No. 6 tube 2 c.c. 0.85 S.S.S. (7) Keep 24 hr. at 37°C. (8) Note the degree of agglutination in each tube. (9) Centrifugalize at high speed. (10) Use the clear S.N.F. of each tube in test, undiluted and in dilutions 1—2, 3 and 4. (11) Set out 3 racks of 5 rows each, for 4 small T.T. in each row. (12) Add to each T.T. of each row of No. 1 rack 0.5 c.c. of diluted and undiluted S.N.F. from No. 6 tube, one dilution to each T.T. of a row. (13) Add to the T.T. of No. 2 rack 0.5 c.c. of diluted and undiluted S.N.F. from centrifuge tubes Nos. 1 to 5, one S.N.F. only with its dilutions to one row. (14) Proceed with No. 3 rack in identical fashion to No. 2 rack. (15) Add to each T.T. of row 1 No. 1 rack and to each T.T. of row 1 No. 3 rack 0.5 c.c. antigen suspension homologous type meningococcus. (16) Add to each T.T. of rows 2, 3, 4 and 5, respectively, of Nos. 1 and 3 racks 0.5 c.c. antigen suspension, test, first heterologous type, second heterologous type, and third heterologous type meningococcus, one of these suspensions for each row of each rack. (17) Add to each T.T. of all rows of No. 2 rack 0.5 c.c. antigen suspension homologous type meningococcus throughout.

**Notes.**—<sup>1</sup>The test consists in a determination of the agglutinating potency of an unabsorbed type serum with regard to type 1, test, type 2, type 3 and type 4 meningococci, and of its potency after absorption by type 1, test, type 2, type 3 and type 4 meningococci to its homologous type meningococcus, and of its potency after absorption by its homologous type meningococcus to type 1, by test meningococcus to test meningococcus, by type 2 meningococcus to type 2, by type 3 meningococcus to type 3, and by type 4 to type 4. Rack No. 1 gives the agglutination of meningococci, with the selected unsaturated serum. Rack No. 2 controls the saturation of the serum by homologous, heterologous and test meningococci. Rack No. 3 gives the degree to which the various meningococci remove any secondary (group) agglutinins contained in the selected serum. <sup>2</sup>Before proceeding to a saturation test each test meningococcus is provisionally typed by agglutination and the type serum or sera selected for the tests on the result of this agglutination. <sup>3</sup>What the three heterologous types will be depends on what is the type serum selected as a result of agglutination tests. <sup>4</sup>The arrangement given is based on the existence of 4 type meningococci.

**B8-322.**—(1) Set up a series of T.T. containing the amount of anti-serum which is used in test. (2) Determine the amount of homologous organism antigen which serves just to reduce the agglutinating power of the serum for the homologous organism to nil or nearly nil and use this amount in test. (3) Set up antigen anti-serum mixtures. (4) Keep the mixtures in the ice chest over night. (5) Centrifugalize. (6) Test S.N.F. for agglutinating power to homologous, heterologous and test antigen.

**BS-33 ERYTHROCYTES.<sup>1</sup>**

**Notes.**—Used in testing the suitability of a donor's blood for transfusion. Besides the undesirable agglutination of erythrocytes an undesirable hemolysis (B 10-42) of erythrocytes may be found.

**BS-331.**—(1) Collect donor's and patient's erythrocytes<sup>1</sup> by taking the blood into 1 per cent citrated 0.95 per cent salt sol. (2) Make a 1:20 dilution of the citrated blood. (3) Set up a series of 4 small T.T., No. 1 containing in equal quantities patient's serum and patient's erythrocytes, No. 2 donor's serum and patient's erythrocytes, No. 3 patient's serum and donor's erythrocytes, No. 4 donor's serum and donor's erythrocytes. (4) Examine for agglutination.

**Notes.**—<sup>1</sup>The erythrocytes may be washed, although this is not absolutely necessary.

**BS-332<sup>1</sup>.**—(1) Have available, if possible, a group 2 and group 3 standard blood serum. (B10-4221). (2) Prepare mixtures on coverslips of one loopful of each serum and one loopful of each erythrocyte suspension. (3) Prepare hanging drops. (4) Ring the coverslips with vasoline. (5) Keep at R.T. (6) Examine every 5 min. with a  $\frac{2}{3}$  in. objective taking care to differentiate between rouleaux formation and clumping.

**Notes.**—<sup>1</sup>The point of importance to determine is that the serum of the patient shall not agglutinate the erythrocytes of the donor. The erythrocytes of group 1 are not agglutinated by serum of groups 2 and 3. The erythrocytes of group 2 are agglutinated by group 3 serum and not by group 2 serum. The erythrocytes of group 3 are agglutinated by group 2 serum and not by group 3 serum. The erythrocytes of group 4 are agglutinated by group 2 and group 3 sera. Thus by the use of serum from individuals of group 2 and 3, all bloods can be grouped. Group 1 donors can give blood to anybody; group 1 donors can only give blood to group 4 patients; group 2 can give to groups 1 and 2 and group 3 to groups 1 and 3. It is best, if possible, to use only group 1 donors. Group 2 and group 3 sera can be preserved in the ice chest for several months. Group 2 and group 3 erythrocytes may be preserved for 4 weeks in the ice chest in the following sol.:—3 drops blood to each c.c. of the mixture,—formalin 9.5; 2 per cent citrated 0.85 S.S.S. 5000. Agglutination should take place within 5 min. The classification of groups here used is the Jansky classification which, stated in full, is as follows:—

**Group 1:**—The serum of this group agglutinates the erythrocytes of groups 2, 3 and 4, while the erythrocytes of this group are not agglutinated by any serum.

**Group 2:**—The serum agglutinates the erythrocytes of groups 3 and 4, but not those of groups 1 and 2, while the erythrocytes are agglutinated by the serum of groups 1 and 3, but not by those of groups 2 and 4.

**Group 3:**—The serum agglutinates the erythrocytes of groups 2 and 4, but not those of groups 1 and 3, while the erythrocytes are agglutinated by the serum of groups 1 and 2, but not by those of groups 3 and 4.

**Group 4:**—The serum agglutinates the erythrocytes of no group while the erythrocytes are agglutinated by the serum of groups 1, 2 and 3.

Another classification which has been much used is the Moss classification, which, stated in full, is as follows :—

*Group 1* :—The serum of this group agglutinates the erythrocytes of no group, while the erythrocytes of this group are agglutinated by the serum of groups 2, 3 and 4.

*Group 2* :—The serum agglutinates the erythrocytes of groups 1 and 3, while the erythrocytes are agglutinated by the serum of groups 3 and 4.

*Group 3* :—The serum agglutinates the erythrocytes of groups 1 and 2, while the erythrocytes are agglutinated by the serum of groups 2 and 4.

*Group 4* :—The serum agglutinates the erythrocytes of groups 1, 2 and 3, while the erythrocytes are not agglutinated by any serum.

#### **B8·4 ANAPHYLACTIC ACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup>Under this term I have included a variety of tests which have for their basis the occurrence of a specific reaction in a sensitized or susceptible individual or the occurrence of a definite train of symptoms.

#### **B8·41 ANAPHYLACTIC SHOCK.<sup>1</sup>**

**Notes.**—<sup>1</sup>The symptoms are :—Restlessness and hyperalgæsia, lowering of temperature, fall of blood pressure, dyspnœa, inspiratory distension, collapse with passage of urine and fæces, stoppage of respiration before cessation of heart's action. Other signs are distension of the lungs and fluidity of the blood.

**B8·411.** (1) Inject a G.P. with 0·01 c.c.<sup>1</sup> foreign serum subcutaneously or intraperitoneally as sensitizing dose. (2) Inject 15 days, 6 months, or 12 months later, 0·25 c.c. of the same foreign serum intravenously, intraspinally, or subdurally or 5 to 10 c.c. subcutaneously as shock dose. (3) Observe the symptoms.

**Notes.**—<sup>1</sup>A larger amount such as 5 to 10 c.c. may be used as a sensitizing dose, but in that case the anaphylactic state will take longer to establish.

**B8·412.**—(1) Heat milk 20 min. at 100C. (2) Inject 1 c.c. intraperitoneally in a 350-grm. G.P. (3) Inject 0·25 c.c. as shock dose subdurally 20d. or more after the sensitizing dose.

**B8·413.**—(1) Inject a G.P. with 0·01 c.c. egg white subcutaneously. (2) Inject 0·02 c.c. as shock dose<sup>1</sup> intravenously or subdurally 20d. or more after the sensitizing dose.

**Notes.**—<sup>1</sup>An unsensitized G.P. can be given a dose of 1 or 2 c.c. without showing symptoms.

**B8·414.**—(1) Inject a G.P. intraperitoneally with 1 c.c. horse or ox serum as sensitizing dose. (2) Inject 15d. later intravenously 2 c.c. of the serum per kilogram. weight as shock dose.

**B8·415.**—(1) Inject a 1500-grm. rabbit with 5 c.c. foreign serum per kilogram. weight, subcutaneously or intravenously. (2) Inject 10d. later 3 c.c. of the serum intravenously. (3) Observe the result.<sup>1</sup>

**Notes.**—<sup>1</sup>The symptoms of shock should appear in 30 min., and death take place in a few hrs. If the dose is insufficient to produce this effect only a specific local reaction will take place, with local oedema and necrosis.

**B8-416.**—(1) Inject a rabbit subcutaneously with 2 c.c. horse or ox serum as sensitizing dose. (2) Inject 15*l.* later intravenously 2 c.c. of the serum per kilogram, weight as shock dose.

**B8-417.**—(1) Inject a young cat with 0.25 c.c. horse serum. (2) Observe the result.<sup>1</sup>

**Notes.**—<sup>1</sup>Horse serum is markedly toxic, even in the unsensitized animal.

**B8-418.**—(1) Inject a dog subcutaneously with 10 c.c. horse serum. (2) Inject 5 c.c. serum intravenously 5 weeks later. (3) Observe the result.

**B8-42 PASSIVE ANAPHYLAXIA: B8-421.**—(1) Inject a series of 250-grm. G.P. intraperitoneally or intravenously each with 1 c.c. of the serum of a rabbit which has been highly immunized to a given protein antigen. (2) Inject 24 hr. later in the G.P. a series of doses of antigen intravenously to determine the smallest dose which causes death<sup>1</sup> or causes definite symptoms.<sup>2</sup>

**Notes.**—<sup>1</sup>Minimum lethal dose. <sup>2</sup>Minimum symptomatic dose. A measure of the potency of the antiserum.

**B8-422.**—(1) Inject a series of 250-grm. G.P. intraperitoneally with 0.1, 0.3, 0.5, 1 and 2 c.c. of an anti-serum. (2) Inject 24 hr. later each G.P. intravenously with 0.2 c.c. of the protein antigen to the anti serum. (3) Observe the result.

**B8-423.**—(1) Mix 1 c.c. of anti-serum with a series of increasing quantities of its protein antigen. (2) Inject the mixtures intraperitoneally in 250-grm. G.P. (3) Determine the degree of anaphylaxis by injecting a large dose of antigen intravenously.

**B8-424.**—(1) Inject a rabbit 2 or 3 times, with several days interval, subcutaneously, intraperitoneally, or intravenously with serum, milk, or other protein antigen. (2) Bleed the rabbit 6*l.* after the last injection. (3) Inject a G.P. with 1 c.c. of the serum obtained. (4) Inject the G.P. 24 hr. later with 0.5 c.c. protein antigen.

**B8-425.**—(1) Keep rabbit anti-serum 18 hr. on ice in contact with a suitable amount of its protein antigen.<sup>1</sup> (2) Centrifuge. (3) Remove the S.N.F. and keep 15 hr. at 37C. (4) Inject 0.05 c.c. of this S.N.F. into the shaven skin<sup>2</sup> of a G.P., or 0.5 c.c. intravenously.<sup>3</sup>

**Notes.**—<sup>1</sup>*L. y.* foreign serum, bacteria, tumour tissue, placenta, tumour, etc. An agglutination should be produced if the anti-serum contains antibodies to the protein antigen. <sup>2</sup>Anaphylactic shock should be produced if the anti-serum contains antibodies to the protein antigen, otherwise injection even of 5 cc. is without effect.

**B8\*43 BACTERIA.**—(1) Inject G.P. with 0.01 mgm. killed<sup>1</sup> bacteria in suspension subcutaneously or intraperitoneally on 6 to 10 successive days. (2) Inject 15d. later 1 to 2 c.c. of a thick<sup>2</sup> suspension of the bacteria intravenously. (3) Observe the symptoms.

**Notes.**—<sup>1</sup>By heat. <sup>2</sup>One agar slope culture in 10 to 15 c.c. N-10 sod. hydroxide.

**B8\*44 ANAPHYLACTIC EFFECT WITHOUT ANTI-SERUM :**

**B8\*441.**—(1) Mix<sup>1</sup> 0.1 c.c. inactivated<sup>2</sup> normal horse serum with 4 to 8 c.c. fresh G.P. serum. (2) Leave 24 hr. (3) Inject 4.5 c.c.<sup>3</sup> of the mixture intravenously in a G.P. (4) Observe the effect.

**Notes.**—<sup>1</sup>The mixture may consist of 0.1 c.c. inactivated normal G. P. serum with 4 to 8 c.c. fresh horse serum. Mixtures of coagulated albumin, horse serum heated to 100C, nutrient agar, peptone, bacteria, etc., with fresh G. P. serum produce the same results. <sup>2</sup>30 min. at 50 to 60C. <sup>3</sup>This quantity should produce fatal symptoms.

**B8\*442.**—(1) Mix bacterial suspension with fresh G.P. serum in the proportion :—suspension 1 ; serum 4. (2) Keep 6 hr. at 37C. (3) Centrifugalize at high speed. (4) Inject the S.N.F. intravenously in the G.P. (5) Observe the effect.

**B8\*443.**—(1) Inject a G.P.<sup>1</sup> intravenously with 6.8 gm. peptone per kilogram weight. (2) Observe the symptoms.

**Notes.**—<sup>1</sup>Rabbits and rats are more resistant than G. P. The M.L.D. for these animals are :—G. P. 0.3 to 0.8 gm., rabbit 3 gm. ; rat 2 gm. per kilogram weight.

**B8\*44.**—(1) Inject a dog intravenously with 0.3 gm.<sup>1</sup> peptone per kilogram weight. (2) Observe the symptoms.<sup>2</sup>

**Notes.**—<sup>1</sup>Doses as small as 0.03 gm. may be sufficient to produce symptoms. <sup>2</sup>Should appear in about 30 sec.

**B8\*45 CUTANEOUS<sup>1</sup> REACTIONS.**

**Notes.**—<sup>1</sup>Under this title are included not only the results of surface applications to the scarified skin, but also intracutaneous, percutaneous and subcutaneous tests.

**B8\*451.**—(1) Shave the skin of a rabbit over flank or abdomen 10 d. after administering the sensitizing dose of protein antigen. (2) Inject 5 hr. after the shaving 0.001 to 0.1 c.c. protein antigen intracutaneously.<sup>1</sup>

**Notes.**—<sup>1</sup>V. B. 8\*473.

**B8\*452.**—V. in detail under tuberculin, mallein and hay fever tests.

**B8\*46 OPHTHALMO REACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup>V. in detail under tuberculin, mallein and hay fever tests. Syn, conjunctival test.

**B8:47 B. TUBERCULOSIS.<sup>1</sup>**

**Notes.**—<sup>1</sup>In addition to the local reaction there may be a general reaction, and local reaction in the neighbourhood of the tuberculosis infection. The general reaction consists in rise of temperature, general malaise, rapid pulse, increased respiration, and loss of appetite.

**B8:471 CUTANEOUS SURFACE TEST.** (1) Disinfect an area on the forearm with alc. and ether. (2) Make 3 abrasions<sup>1</sup> 2 in. apart with a vaccination borer or other suitable instrument. (3) Apply a drop of undiluted old tuberculin to the upper and lower abrasions. (4) Remove the excess of tuberculin with absorbent cotton wool after 5 min. (5) Leave the untouched centre abrasion<sup>2</sup> as a control. (6) Observe the reaction.<sup>3</sup>

**Notes.**—Without causing bleeding. <sup>2</sup>Taking very great care that no tuberculin comes in contact with it. <sup>3</sup>The reaction appears in 4 to 6 hr., reaches its maximum in 24 to 48 hr. and then subsides rapidly. The degree of reaction is given by the diameter of the reddened area. A reaction is considered positive which shows an areola of at least 5 mm. greater diameter than any appearing at the control area.

**B3:472 PERCUTANEOUS TEST.** (1) Disinfect an area<sup>1</sup> on the forearm, chest, or abdomen, with alc. and ether. (2) Rub in with the finger for 1 min. a piece of ointment<sup>2</sup> about the size of a pea over any area not greater than 5 sq. cm. (3) Examine for reaction<sup>3</sup> after 24 and 48 hr.

**Notes.**—<sup>1</sup>The skin should be shaved free of hairs. <sup>2</sup>Equal parts old tuberculin and lanolin. Undiluted tuberculin may be used instead of an ointment. <sup>3</sup>Erythema and a papular eruption.

**B3:473 INTRACUTANEOUS TEST.** (1) Use a syringe with very fine needle. (2) Inject into the cutis on the back of the forearm 0.05 to 0.1 c.c. 1:5000 old tuberculin to produce a raised white area.<sup>1</sup> (3) Repeat the injection if negative with a concentration of 1:1000 and if still negative with 1:100, and finally, if necessary, with 1:10 tuberculin. (4) Examine from the 8th hr. onward for reaction.<sup>2</sup>

**Notes.**—<sup>1</sup>This disappears in about 15 min. <sup>2</sup>The reaction reaches its maximum in 24 to 48 hr. No reaction is considered positive which does not persist for more than 48 hr. The degree of reaction is given by the diameter of the infiltrated area.

**B8:474 INTRACUTANEOUS ANIMAL TEST.** (1) Shave an area at the side of the neck or at the root of the tail. (2) Inject into the skin 0.1 c.c. 50 per cent old tuberculin. (3) Wait 48 hr. (4) Choose 2 points on the shaven surface of the skin exactly 5 cm. apart. (5) Compress the skin between these 2 points with the fingers. (6) Take

the thickness of the compressed skin with calipers and compare<sup>1</sup> with the same skin measured<sup>1</sup> before injection.

**Notes.**—<sup>1</sup>Differences in the thickness up to 0.4 cm. may be considered as negative, 0.5 to 0.7 cm. as doubtful, and swellings of over 7 cm. as distinctly positive.

**B8-475 SUBCUTANEOUS TEST.** (1) Take the patient's temperature<sup>1</sup> and pulse rate 2-hourly for 5*d*. (2) Inject just below the angle of the scapula or below the insertion of the deltoid, or above the ankle 0.2 mgm.<sup>2</sup> old tuberculin. (2) Observe the temperature every 3 hr. (3) Inject 1 mgm. at the end of 48 hr. if there is no reaction, but if there is a reaction, even of half a degree, repeat the first dose only.<sup>3</sup> (4) Give a dose of 5 mgm. as third dose if there is still no reaction. (5) Give a final dose of 10 mgm. as fourth dose before concluding as to freedom from tuberculosis, always supposing there has been no reaction.

**Notes.**—<sup>1</sup>The temperature should not be over 99F. for the test to be applied. <sup>2</sup>0.1 mgm. for children under 15 yr. <sup>3</sup>If the temperature shows irregularity, the intervals between injections should be 72 hr. A rise of one degree Fahrenheit or more above the previous maximum constitutes a positive reaction. Local and focal reactions should be looked for. The local reaction—track reaction—should appear in 24 hr. and consists of subcutaneous infiltration, pain and swelling, and redness at that point where the eye of the needle lay. In cases of great susceptibility the entrance point and the needle track will also show redness.

**B8-476 SUBCUTANEOUS ANIMAL TEST.** (1) Keep the animal in its stall for some days before inoculation. (2) Take the rectal temperature of the animal morning and evening for 2 or 3*d*. previous to inoculation. (3) Inject old tuberculin,<sup>1</sup> suitably diluted, subcutaneously, in the middle of one side of the neck about 11 P.M. (4) Take the temperature of the animal at the time of inoculation and again 6 hr. later. (5) Continue to take the temperature every 2 hr. thereafter up to 24 hr. after inoculation. (6) Record the reaction.<sup>2</sup>

**Notes.**—<sup>1</sup>0.5 to 1 c.c. in horses and oxen, 0.05 to 0.15 c.c. in sheep and goats, 0.01 to 0.1 c.c. in dogs. <sup>2</sup>The reaction consists of a rise of temperature of 1.5 to 6F. It appears in 6 to 8 hr. and reaches its maximum in 15 hr. after injection. There is also some constitutional effect. Healthy animals show practically no temperature reaction. If it is suspected that the animal has had a dose of tuberculin shortly before the test in order to mask the reaction, give double the dose of tuberculin and take the temperature within 3 hr. of injection.

**B8-477 CONJUNCTIVAL TEST.**<sup>1</sup> (1) Examine the eye for freedom from disease. (2) Instil<sup>2</sup> one drop 1 per cent<sup>3</sup> old tuberculin<sup>4</sup> into the conjunctival sac of the lower lid near the inner canthus. (3) Observe the results<sup>5</sup> after 6, 8, 12, 24 and 36 hr. (4) Repeat the

procedure on the other eye, if the result is negative. 2d. Filter with 4 per cent old tuberculin.

**Notes.**—<sup>1</sup>Syn. ophthalmoreaction. Great care should be taken that one or both eyes is subjected to this test. <sup>2</sup>Keep the patient, after the instillation, with head thrown back and eye open for 1 min. <sup>3</sup>0.5 percent in children. <sup>4</sup>It is better to use a tuberculin specially prepared for this test. Such a preparation can be purchased. It can be prepared by precipitating old tuberculin with 20 vol. abs. alc., filtering off the precipitate and drying it *in vacuo* over sulphuric acid or calc. chloride. The dried product should be used as a 4 per cent suspension in D.W. <sup>5</sup>The reaction appears in 6 to 8 hr., reaches its maximum in 24 to 48 hr. and disappears in 4 to 6 d. The degree of the reaction may be described as: grade 1—reddening of the corneal and palpebral conjunctiva; grade 2—intense reddening, with involvement of ocular conjunctiva, swelling and increased secretion; grade 3—intense reddening of the whole conjunctiva, severe chemosis, much fibrinous and purulent secretion and small ecchymoses. <sup>6</sup>1 per cent in children. The reaction is not to be relied on to appear in early tuberculosis. In cattle 20 per cent tuberculin may require to be used.

**B8:478 NASAL TEST.** (1) Use a swab to apply the tuberculin, and keep it pressed against the nasal septum for 10 min. (2) Observe the reaction<sup>1</sup> after 16 and 24 hr.

**Notes.**—<sup>1</sup>Redness, secretion, and formation of crusts.

#### **B8:479 URETHRAL<sup>1</sup> AND VAGINAL<sup>1</sup> TESTS.**

**Notes.**—<sup>1</sup>These may be useful in cattle.

#### **B8:48 B. MALLEI: B8:481 SUBCUTANEOUS ANIMAL TEST.**

(1) Keep the animal in its stall for some days before inoculation. (2) Take the rectal temperature of the animal morning and evening for 2 or 3 d. previous to inoculation. (3) Inject mallein suitably diluted<sup>1</sup> subcutaneously in the middle of one side of the neck at 6 A.M. (4) Take the temperature of the animal every two hr. for at least 18 hr. (5) Take the temperature of the animal at least 3 times on the day succeeding the injection. (6) Record the reaction.<sup>2</sup>

**Notes.**—<sup>1</sup>E.g., 4 c.c. 1-10 mallein, with increase to double if the reaction is negative.

<sup>2</sup>A positive reaction is shown by a rise of temperature within 24 hr., which may be as high as 105F. The temperature has to be carefully watched as it sometimes recedes to normal within a few hr., although it may persist for a day or two. Distent lymphatic swelling at the site of injection may reach a diameter of 20 cm. or more. It is useful to perform a control test on a known healthy animal as a control of technique.

#### **B8:49 B. TYPHOSUS: B8:491 CONJUNCTIVAL TEST.**

Grow a virulent B. typhosus on nutrient agar. (2) Separate the product in D.W. (3) Keep 30 min. at 60C. (4) Centrifuge. (5) Filter the bacterial sediment. (6) Dry *in vacuo* over sulphuric acid and calcium chloride. (7) Grind up 3 grm. dried bacterial mass in an agate mortar

with 1 gm. sod. chloride for 2 or 3 hr., sterile water being added drop by drop until 5 to 6 c.c. have been added. (8) Make up to 100 c.c. with sterile water. (9) Keep 30 min. at 60°C. (10) Decant the S.N.F. from the sediment. (11) Pour the S.N.F. slowly into 10 times its vol. of abs. alc. (12) Collect the precipitate. (13) Dry rapidly *in vacuo* over sulphuric acid or calc. chloride. (14) Use the powder for test dissolved in water in 1 per cent. strength. (15) Instil 1 drop.

**Notes.**—<sup>1</sup>Syn. ophthalmic reaction.

**B8-492 CUTANEOUS SURFACE TEST.**—(1) Use a killed suspension of bacteria for the test.

**B8-5 ANAPHYLACTIC ACTION: E8-51 MENINGOCOCCUS: B8-511 INTRACUTANEOUS TEST.**—(1) Grow a series of strains on 1 per cent<sup>1</sup> starch agar without peptone 48 hr. (2) Suspend the several cultures each in 3 c.c. 0.85 S.S.S. (3) Mix. (4) Add to the mixture 3 times its vol. abs. alc. (5) Centrifugalize. (6) Remove the S.N.F. (7) Shake up the deposit in the same vol. of abs. alc. (8) Centrifugalize. (9) Remove the alc. (10) Resuspend the deposit in ether which has been distilled over sodium. (11) Centrifugalize and decant the S.N.F. (12) Dry the deposit *in vacuo* over sulphuric acid. (13) Grind up to powder. (14) Dry again 24 hr. (15) Make into suspension with 0.5 per cent carbolized 0.85 S.S.S. (16) Inject intracutaneously with a fine needle 0.05 c.c. antigen suspension. (17) Read the reaction<sup>1</sup> at 6, 24 and 48-hr. periods.

**Notes.**—<sup>1</sup>A positive reaction—a well demarcated areola 3 to 7 mm. diameter which is indurated.

**B8-52<sup>1</sup> DIPHTher/Æ.** (1) Dilute a fresh diphtheria toxin so that 0.1 c.c. contains 1-50th M.L.D.<sup>2</sup> for a 250-grm. G.P. (2) Inject 0.1 c.c. of this dilution intracutaneously on the flexor aspect of the forearm to produce a raised white area. (3) Observe the result<sup>3</sup> after 24 to 48 hr.<sup>4</sup>

**Notes.**—<sup>1</sup>To determine susceptibility among contacts with a case. See also **B8-915**.  
<sup>2</sup>The M.L.D. is the smallest dose which on subcutaneous injection will just kill a G.P. after 4d. <sup>3</sup>If the injected toxin is neutralized, no reaction follows. If the injected toxin is not neutralized, there results at the site of inoculation an area of circumscribed redness and oedema. <sup>4</sup>A positive reaction persists for 48 hr. or longer.

**B8-53 HAY FEVER: B8-531 CONJUNCTIVA TEST.**<sup>1</sup>—(1) Add 10 c.c. 5 per cent salt sol. to 10 mgm. appropriate pollen (**A3-52**). (2) Filter after the soluble extracts have been dissolved. (3) Add 7 per cent abs. alc. to the filtrate and use in test. (4) Instil into the conjunctival

sac, initially a 1-20,000 dilution<sup>2</sup> of the testing sol. and then progressively stronger testing sol. if no reaction<sup>3</sup> takes place.

**Notes.**—<sup>1</sup>Syn., ophthalmic-reaction. Made with 0.5 per cent. salt sol. <sup>2</sup>Salt sol. stronger or weaker than this causes irritation independently of the action of the pollen extract. <sup>3</sup>The reaction shows itself as a slight itching, faint reddening at the inner canthus, secretion of tears, and sneezing. The reaction should appear in about 3 min., reach a maximum in about 5 min., and disappear in 30 min. Slightly susceptible individuals may require a testing sol. 10 to 20 times stronger than in very susceptible individuals to elicit a reaction. Normal individuals will scarcely react even to freshly dried pollen. The minimal reacting dose is made the basis of active immunization against hay fever.

**B8-532 ANTITOXIN CONJUNCTIVAL TEST.** (1) Use as standard dose<sup>1</sup> one drop 1-40,000 rye pollen protein sol. equivalent to 1-1000th mgm. dry protein. (2) Set up mixtures containing varying dilutions of antitoxin with 1-40,000 pollen sol. all with the same total vol. (3) Keep 1 hr. at 37°C. (4) Determine which of these mixtures<sup>2</sup> on instillation into the conjunctival sac of a hay fever patient just fails to give a reaction.

**Notes.**—<sup>1</sup>This quantity of pollen extract should definitely produce reaction in a susceptible individual. <sup>2</sup>The dilution of the serum at this point is the index to its potency.

**B8-533 CUTANEOUS TEST.**<sup>1</sup> (1) Scarify a convenient portion of the skin to remove the dead horny layers. (2) Apply to this area a watery extract of pollen or pollen powder itself.

**Notes.**—<sup>1</sup>Can be used in a patient actually suffering from hay fever.

**B8-54 SPIROCHÆTA PALLIDA ; B 8-541 INTRACUTANEOUS TEST.**—(1) Grow the *Sp. pallida* in cecitic fluid tissue agar anaerobically under an oil seal. (2) Cut the tube and remove the agar. (3) Cut out the portion showing most marked spirochæte growth. (4) Grind up this portion in a sterile mortar to a paste and add to it a fluid culture of spirochætes so as to produce a uniform suspension. (5) Keep 1 hr. at 60°C. (6) Add 0.5 per cent phenol. (7) Test sterility. (8) Use in test. (9) Dilute the antigen suspension with an equal vol. of 0.85 S.S.S. (10) Inject as dose for an adult 0.07 c.c. intracutaneously at the insertion of the deltoid muscle. (11) Note the reaction.<sup>1</sup>

**Notes.**—<sup>1</sup>A positive reaction appears in 36 hr. and does not subside till the 4th day. A negative traumatic reaction may also show itself but disappears within 4 hr.

**B8-6 ANTIANAPHYLATIC ACTION.**<sup>1</sup>

**Notes.**—<sup>1</sup>Desensitization.

**B8·61 SUBCUTANEOUS : B8·611.** (1) Inject a G.P. intravenously with 0·25 c.c. foreign serum as sensitizing dose. (2) Inject 0·01 c.c. serum subcutaneously at hourly intervals 14*d.* later. (3) Watch very carefully for symptoms after each injection. (4) Give a shock dose of 0·5 c.c. serum intravenously soon after the last subcutaneous injection. (5) Observe the symptoms<sup>1</sup> produced.

**Notes.**—<sup>1</sup>The symptoms of anaphylaxis will not appear, or will be very much less than when the shock dose is administered 14*d.* after the sensitizing dose without any previous desensitizing doses.

**B8·612.**—(1) Inject in man 0·5 c.c. horse serum subcutaneously 4 hr. before the injection of antitoxic serum, in case he may have had a previous injection of antitoxin which could render him anaphylactic.

**B8·613.**—(1) Inject subcutaneously as a desensitizing dose 1-10th to 1-20th of a full dose of bacterial antigen 10 min. before the administration of the latter.

**B8·62 INTRAVENOUS : B8·621.**—(1) Inject a sensitized G.P. intravenously with 0·025 c.c.<sup>1</sup> serum. (2) Inject 0·1 c.c. 5 min. later. (3) Inject 0·25 c.c. 2 min. later.

**Notes.**—<sup>1</sup>The shock dose being 0·05 c.c.

**B8·622.**—(1) Inject a rabbit<sup>1</sup> which is being used to produce hæmolytic serum intravenously with a small dose, such as 0·2 to 0·5 c.c. blood the evening before or even 10 min. before the full regular dose.

**Notes.**—<sup>1</sup>Many of these rabbits die in the course of immunization if not thus treated.

**B8·63 INTRAPERITONEAL : B8·631.**—(1) Inject a sensitized G.P. intraperitoneally with 3 c.c. serum heated at 80C. to make it resistant to a fatal subdural shock dose.

**B8·632.**—(1) Inject a sensitized G.P. with 0·02 c.c. unheated horse serum intraperitoneally or 0·05 c.c.<sup>1</sup> subcutaneously in order to protect it from an intravenous or subdural shock dose administered 4 hr. later.<sup>2</sup>

**Notes.**—<sup>1</sup>A dose at least 50 times less than the fatal dose. <sup>2</sup>Desensitization on the average may be set down as established 4 hr. after the desensitizing dose when administered subcutaneously; 1½ hr. intraperitoneally.

**B8·64 SUBDURAL : B8·641.**—(1) Inject 0·25 c.c. serum subdurally before the completion of the incubation period<sup>1</sup> in a sensitized animal.

**Notes.**—<sup>1</sup>12*d.*

**B8\*642.** (1) Inject less than the shock dose<sup>1</sup> of serum subcutaneously in the sensitized animal if the incubation period<sup>2</sup> has been completed.

**Notes.**—<sup>1</sup>0.002 to 0.025 c.c. 242 d.

**B8\*65 ORAL : B3.651.**—(1) Give a G.P. sensitized to white of egg 5 c.c. of white of egg to ingest 2 or 3d. before administering the shock dose.

**B8\*66 RECTAL : B3\*661.** (1) Inject per rectum 7.5 c.c. serum in the sensitized animal.

#### **B8\*7 ANTIENDOTOXIC<sup>1</sup> ACTION.**

**Notes.**—<sup>1</sup>In which is included protective and antibacterial action.

**B8\*71 V. CHOLERÆ : B8\*711.**—(1) Add diminishing quantities of antiendotoxic serum to a series of T.T. containing 2 M.L.D. toxin. (2) Keep 10 min. at R.T. (3) Inject the mixture subcutaneously in a 250-grm. G.P. (4) Determine the amount of serum necessary to neutralize the toxin.

**B8\*712.** (1) Inject a series of mice subcutaneously with 10 M.L.D. of a serum broth culture. (2) Inject 20 hr. later, intraperitoneally, varying doses of anti-serum, to determine the smallest dose which will protect.

**B8\*72 B. DYSENTERIÆ SHIGA : B8\*721.**—(1) Determine the intravenous minimum lethal dose of toxin for the rabbit. (2) Set up a series of T.T. each containing 10 minimum lethal doses of toxin with diminishing quantities of anti-serum. (3) Bring the total vol. of the mixtures up to 4 c.c. with 0.85 S.S.S. (4) Keep 1 hr. at 37°C. (5) Inject the mixtures intravenously in young rabbits. (6) Observe the animals for at least 5d. (7) Determine the protective power of the test serum.

**Notes.**—<sup>1</sup>The symptoms of toxic action are diarrhoea, paralysis of extremities, loss of weight, and death.

**B8\*722.**—(1) Inject 200-grm. G.P. intraperitoneally with 0.5, 0.3, 0.1, . . . . . c.c. anti-endotoxic serum. (2) Inject a minimum lethal dose of endotoxin 6 hrs. later.

**B8\*723.**—(1) Inject 200-grm. G.P. intraperitoneally with one M.L.D. of endotoxin. (2) Inject anti-serum intraperitoneally 1 hr. later in quantities of 0.1, 0.3, 0.5 and 1 c.c.

**B8\*724.**—(1) Mix the 24-hr. intraperitoneal M.L.D. of serum with diminishing quantities of anti-serum in a T.T. (2) Inject the mixture intraperitoneally in G.P.

**B8\*725.**—(1) Select an antidyenteric serum to be the standard. (2) Preserve in a dry state *in vacuo*. (3) Use as unit the amount of standard antitoxin which neutralizes 100 M.L.D. of a given toxin. (4) Standardize test sera by means of the L+ dose<sup>1</sup> of the toxin, using rabbits as the test animal, intravenous dosage, and survival or death in 4 or 5d. as the criteria.

**Notes.**—<sup>1</sup>The toxin antitoxin mixture is kept 1 hr. at 37C. before injection.

**B8\*73 B. TYPHOSUS : B8\*731.**—(1) Determine the 24-hr. intraperitoneal M.L.D. of *B. typhosus* for a 250-grm. G.P. (2) Mix 10 M.L.D. with diminishing quantities of an antityphoid serum. (3) Determine the amount of serum which just suffices to protect a G.P. against this quantity of antigen—a protective unit of serum. (4) Determine the L+ dose<sup>1</sup> of the culture with reference to the unit of serum. (5) Titrate<sup>2</sup> test sera against this L+dose.

**Notes.**—<sup>1</sup>*Y.* **B8\*9138.** and **S7\*4103.** <sup>2</sup>The amount of test serum which just permits the death of the G.P. within 24 hr. contains one protective unit.

**B8\*74 MENINGOCOCCUS : B8\*741 ANTIGEN.**—(1) Grind up 0.05 gm. dried meningococcus in an agate mortar with 1.25 c.c. D. W. (2) Add 1.25 c.c. N-20 sod. hydroxide.

**B8\*742 TEST.**—(1) Set up mixtures of graded quantities of antigen 0.1 to 0.15 c.c. with 0.5 c.c. normal and test sera. (2) Keep 30 min. at 37C. (3) Inject the mixtures intraperitoneally into mice of 12 to 20 gm. weight. (4) Keep the animals under observation in a warm<sup>1</sup> place.

**Notes.**—<sup>1</sup>The endotoxin produces a fall of temperature.

**B8\*75 B. ANTHRACIS : B8\*751.**—(1) Inject 5 rabbits intravenously with 6, 5, 4, 3 and 2 c.c. anti-serum. (2) Inject 5 to 10 min. later these rabbits subcutaneously each with 0.002 mgm. virulent *B. anthracis*. (3) Inject a control rabbit with 6 c.c. normal serum followed by 0.002 mgm. virulent *B. anthracis*. (4) Determine the quantity of serum which protects.

**B8\*752.**—(1) Determine the quantity<sup>1</sup> of anti-serum which in mixture with 1000 M.L.D. of *B. anthracis* can delay the death of a rabbit subcutaneously injected for 6 to 8 hr. as compared with a control rabbit.

**Notes.**—<sup>1</sup>This amount is described as representing half a unit.

**B8\*8 ANTIHÆMOLYTIC ACTION : B8\*81 BACTERIAL HÆMOLYSIN : B8\*811.**—(1) Set up a series of small T.T. containing 2 c.c.

1 per cent suspension of erythrocytes (**A3\*411**). (2) Allow the erythrocytes to sediment.<sup>1</sup> (3) Add diminishing quantities 0.5, 0.2, 0.1, ..., 0.001 c.c. of bacterial hæmolysin (**B8\*812**) to each T.T. (4) Bring the total vol. up to 3 c.c. by addition of 0.85 S.S.S. (5) Shake gently to mix. (6) Keep 2 hr. at 37°C. (7) Determine the minimum hæmolytic dose of toxin. (8) Set up a series of small T.T. containing diminishing quantities 1, 0.5, 0.1, ..., 0.001 c.c. inactivated anti-serum. (9) Add the minimum hæmolytic dose of bacterial hæmolysin to each T.T. (10) Set up control mixtures. (11) Bring all the mixtures up to the same vol. with 0.85 S.S.S. (12) Keep 30 min. at 37°C. (13) Add to each T.T. 2 c.c. erythrocyte suspension. (14) Keep 2 hr. at 37°C. and 2 hr. at R.T. (15) Read results of antihæmolytic action.

**Notes.**—<sup>1</sup>This allows of the admixture of toxin and fluid, before the toxin can come into contact in more concentrated condition with the erythrocytes.

**B8\*812.**—(1) Grow the hæmolysin-producing organism 3 weeks in bouillon. (2) Filter the bouillon through a Berkefeld candle. (3) Add 0.5 per cent phenol to the filtrate. (4) Preserve this bacterial hæmolysin in a cool dark place. (5) Determine the smallest amount of bacterial hæmolysin which can completely lyse 1 c.c. 1 per cent suspension washed rabbit erythrocytes in 1 hr. at 37°C. (6) Set up a series of small T.T. each containing the minimum hæmolytic dose of hæmolysin. (7) Add 1 c.c. serial dilutions of test serum<sup>1</sup> and normal serum. (8) Bring all the mixtures up to the same vol. with 0.85 S.S.S. (9) Keep 30 min. at 37°C. (10) Add to each T.T. 1 c.c. 1 per cent suspension washed rabbit erythrocytes. (11) Keep 2 hr. at 37°C. and 2 hr. at R.T. (12) Determine<sup>2</sup> the quantity of test serum sufficient to neutralize the hæmolysin and compare it with that of normal serum.

**Notes.**—<sup>1</sup>A high antihæmolytic value of test serum for staphylococcal hæmolysin is said to indicate a staphylococcal infection. <sup>2</sup>The limit of antihæmolytic power of the serum is shown by the T. T. containing the smallest quantity of serum which completely prevents hæmolysis, that is to say, by the last T. T. in which the S. N. F. remains absolutely colourless.

### **B8\*9 ANTITOXIC ACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup>Consult also S7\*4 and A3\*24.

**B8\*91 B. DIPHTHERIÆ : B8\*911.**—(1) Determine the M.L.D. for a G.P. (2) Calculate<sup>1</sup> the number of grm. of G.P. which can be protected by 1 c.c. antitoxin.

**Notes.**—<sup>1</sup>Thus, for example, if 0.01 c.c. serum just protects a 100-unit G.P. the value of the serum will be  $100 \times 450 = 45,000$ .

**B8\*192**<sup>1</sup> (1) Use 10 M.L.D., instead of 1 M.L.D., as in **B8\*911**.

**Notes.** <sup>1</sup> More used for the standardization of tetanus antitoxin and of antivenom than for diphtheria.

**B8\*913.** (1) Use a stable<sup>1</sup> and potent<sup>2</sup> toxin. (2) Determine the M.L.D.<sup>3</sup> (3) Dilute standard serum<sup>4</sup> with glycerin 2 : 0.85 S.S.S. 1 to contain 10 units per c.c. (4) Dilute again for use 1-10 with 0.85 S.S.S. to contain 1 antitoxin unit per c.c. (5) Set up a series of T.T. each containing 1 unit<sup>5</sup> of standard antitoxin. (6) Add varying quantities<sup>6</sup> of toxin to the antitoxin in each T.T. (7) Bring the total vol. of each of the mixtures up to 4 c.c. with 0.85 S.S.S. (8) Keep the mixtures 30 min. at R.T. (9) Inject the mixtures subcutaneously into the abdominal wall of 250 to 280-grm. G.P. (10) Weigh the G.P. daily. (11) Note the condition of the site of inoculation as regards swelling. (12) Determine the Lo and L+ doses<sup>7</sup> of toxin from the results. (13) Use the L+ dose thus determined to test the antitoxin content of test sera. (14) Make dilutions of the test anti-serum according to its probable strength. (15) Mix 2 c.c. of each dilution with the L + dose of toxin. (16) Bring the total vol. of each mixture up to 4 c.c. with 0.85 S.S.S. (17) Keep the mixture 30 min. at R.T. (18) Inject the mixtures subcutaneously into a series of 250 to 280-grm. G.P. (19) Note the condition of the site of inoculation as regards swelling. (20) Determine the Lo and L+ doses of toxin in these mixtures with test anti-serum. (21) Make a P.M. of the G.P. to exclude death from concurrent disease. (22) Calculate<sup>8</sup> the potency of the test anti-serum.

**Notes.** —<sup>1</sup> The toxin after preparation should be kept in a cool dark place in closely stoppered 2-litre bottles under a layer of toluol. It may be 9 to 12 m. after being freed by filtration from bacteria (**A3\*241**) before this stability is established. The standard stable toxin must be tested every 6 weeks against the standard serum. <sup>2</sup>For example, fatal on subcutaneous injection in the G.P. in a dose of 0.005 c.c. <sup>3</sup>The dose of toxin which on subcutaneous injection causes the death of 250 to 280-grm. G.P. in 4 d. <sup>4</sup>Very special precautions are taken in the state laboratories to which this task is assigned for the preservation of the standard antitoxin in a dried state. <sup>5</sup>The unit is an arbitrary one. The unit of standard antitoxin of Ehrlich is that quantity of antitoxic serum which at a given time neutralized 100 M.L.D. of a particular *pure* diphtheria toxin. A *pure* toxin is a fresh toxin and is defined as one containing only toxin and no toxoid or other constituent capable of uniting with antitoxin to neutralize it. The antitoxin carefully dried and preserved in a desiccated condition is a stable substance and serves as a standard of measurement. <sup>6</sup>As the object of the trial is to determine the dose of toxin which is just neutralized by one unit of standard antitoxin and also the dose which is neutralized to the extent of leaving sufficient toxin to kill the G.P. on the 4th day it is obvious that the amount of toxin to be used in these mixtures must be adjusted by its power of combining with 1 unit of antitoxin. The varying quantities used may suitably vary by

ant. i.e. <sup>2</sup>See also **B7-4103**. The L<sub>0</sub> dose of toxin is that quantity which when mixed with 1 unit of standard antitoxin produces neither general nor local toxic action upon subcutaneous injection in a 250 to 280-grm. G.P. The L<sub>+</sub> dose of toxin is that quantity which when mixed with 1 unit of standard antitoxin causes the death of 250 to 280-grm. G.P. in 4d. after subcutaneous injection. L=limit, limiting quantity. <sup>3</sup>Thus, for example:—If in the series used the injection of 1.550th c.c. test serum mixed with the L<sub>+</sub> dose of toxin results in death on the 4th day while the injection of 1.600th c.c. test serum mixed with the L<sub>+</sub> dose of toxin results in death on the 3rd day, the conclusion follows that the test serum contains at least 350 units but less than 600 units per c.c.

**B8-914.**—(1) Inject a G.P. intracutaneously with a definite fraction of a M.L.D. of toxin mixed with a definite amount of test serum. (2) Set up controls. (3) Observe reaction.<sup>1</sup>

**Notes.**—<sup>1</sup>If the injection is not followed by necrosis of the skin, the toxin has been neutralized by the serum.

**B8-915.**<sup>1</sup>—(1) Make a dilution of fresh standard diphtheria toxin of such strength that 0.1 c.c. contains a definite fraction, 1-20th to 1-50th, of the M.L.D. for a 250 to 280-grm. G.P. (2) Inject intracutaneously on the flexor aspect of the forearm of the suspected individual.<sup>2</sup> (3) Examine for reaction (**B8-5203**).

**Notes.**—<sup>1</sup>V. also **B8-52**. <sup>2</sup>For the detection among contact cases of susceptible individuals.

**B8-92 B. TETANI : B8-921.**<sup>1</sup>—(1) Set up a series of T.T. containing 1 c.c. of a sol. of 0.1 gm. standard<sup>2</sup> dried toxin in 166 c.c. 0.85 S.S.S.<sup>3</sup> (2) Add diminishing quantities of test antitoxin serum to this series. (3) Inject the mixtures subcutaneously in a series of 350-grm. G.P. (4) Determine which of the mixtures contains just sufficient free toxin to kill a 350-grm. G. P. in 4d. (5) Calculate the number of units<sup>4</sup> contained in the test serum.

**Notes.**—<sup>1</sup>United States Hygiene Laboratory method. <sup>2</sup>The toxin in the case of tetanus is more stable than the antitoxin. It is the toxin therefore which is preserved as a standard. The L<sub>+</sub> dose of this standard toxin is fixed at 100 M. L. D. for a 350-grm. G. P. <sup>3</sup>This amount of solvent is used because 1.16666th-grm. (0.00066 gm.) of the toxin selected as standard=100 M. L. D. for a 350-grm. G. P. <sup>4</sup>The unit adopted is 10 times the least amount of serum necessary to save the life of a 350-grm. G. P. for 4d. against the official test dose or L<sub>+</sub> dose of standard toxin. The L<sub>+</sub> dose of standard toxin is made available to manufacturers desirous of testing their antitoxin serum.

**B8-93 B. BOTULINUS : B8-931.**—(1) Determine the amount of toxin which kills a 250-grm. G.P. with certainty in about 48 hr. (2) Determine the amount of anti-serum which renders this dose of toxin harmless. (3) Calculate the number of doses which are neutralized by 1 c.c. of anti-serum.

**B8-94 B. WELCHII.<sup>1</sup>**

**Notes.**—<sup>1</sup>Syn. *B. aerogenes capsulatus*. *B. perfringens*.

**B8-941.<sup>1</sup>**

**Notes.**—<sup>1</sup>Take as unit twice the smallest amount of serum necessary to neutralize 2 M.L.D. toxin injected intramuscularly in a 20-grm. mouse, the mixture of toxin and serum being kept in contact for 1 hr. at R. T. before injection.

**B8-95 V. SEPTIQUE: B. 8-951.<sup>1</sup>**

**Notes.**—<sup>1</sup>Take as unit the smallest amount of serum necessary to neutralize 1 M. L. D. toxin injected intravenously in a full grown rabbit, the mixture of toxin and serum being kept in contact for 1 hr. at R.T. before injection. In practice, a multiple of the M.L.D. is used in the toxin antitoxin mixture.

**B8-96 B. OEDEMATIENS: B8-961.<sup>1</sup>**

**Notes.**—<sup>1</sup>Take as unit the smallest amount of serum necessary to neutralize 100 M.L.D. toxin injected intramuscularly in a 20-grm. mouse, the mixture of toxin and serum being kept in contact for 1 hr. at R.T. before injection.

**B8-97 ABRIN: B8-971.<sup>1</sup>**—(1) Determine the quantity of abrin toxin which kills a white mouse with certainty on subcutaneous injection. (2) Determine the amount of anti-serum which, in mixture<sup>2</sup> with this dose of abrin toxin, renders it harmless on subcutaneous injection in the white mouse.

**Notes.**—<sup>1</sup>The same procedure applies to anti-croton and anti-ricin. <sup>2</sup>The mixture should be kept for 30 min. before injection.

**B8-98 HAY FEVER: B8-981.**—(1) Determine the smallest quantity of pollen or other hay fever antigen (**A3-52**) which elicits the characteristic hay fever symptoms on installation into the conjunctival sac of a susceptible individual. (2) Determine the amount of anti-serum to be added to this dose of antigen to abolish its effect upon the conjunctiva.

**Notes.**—<sup>1</sup>The mixture should be kept for 1 hr. at 37C. before being tested. See also **B 8-532**.

**B9 BLOOD SERUM REACTIONS: B9-1 ANTITRYPTIC ACTION: B9-11 CASEIN-ACETIC METHOD: B9-111 TRYPSIN.**

—(1) Prepare:—pure trypsin powder 0.5; 0.85 S.S.S. 50; N-1 sod. hydroxide 0.5. (2) Dilute the mixture 1-10 with 0.85 S.S.S.

**B9-112 CASEIN.**—(1) Dissolve 1 grm. pure casein in 100 c.c. N-10 sod. hydroxide with gentle heat. (2) Neutralize to litmus with N-10 hydrochloric acid. (3) Make up to 500 c.c. with 0.85 S.S.S. (4) Filter. (5) Sterilize (**S9-5**).

**B9-113 ACETIC ACID.**—(1) Prepare 100 c.c. glacial acetic acid 1 c.c. in 9 c.c. water 10.

**B9-114 SERUM.** (1) Collect the test blood before breakfast. Use the serum, which must be free from haemoglobin, not later than 8 hr. after the collection of blood. (3) Dilute the test serum for use 1-50 with 0-85 S.S.S.

**Notes.** <sup>1</sup>To avoid excess of amino-acids in the blood.

**B9-115 TRYPSIN STANDARDIZATION.** (1) Set up 10 small T.T. containing 2 c.c. casein sol. in each. (2) Add to the first 9 T.T. in serial order decreasing amount, 1, 0-9, 0-8 to 0-2 c.c. trypsin sol. and no trypsin sol. to No. 10 T.T. (3) Bring the vol. in each T.T. up to 3 c.c. with 0-85 S.S.S. (4) Shake gently to mix. (5) Keep 30 min. at 50°C. in a water bath. (6) Add 4 drops ascitic acid sol. to each T.T. and observe which T.T. mixture first shows the cloudiness due to incomplete digestion of casein. (7) Determine the smallest amount of trypsin which gives complete digestion of casein—complete digesting dose.

**B9-116 TEST.**<sup>1</sup> (1) Set up 6 small T.T. containing 0-5 c.c. 1-50 test serum. (2) Add to No. 1 T.T. the complete digesting dose of trypsin. (3) Add to No. 2 T.T. and successive T.T. increasing amount of trypsin so that No. 6 T.T. shall contain 4 times as much as No. 1 T.T. (4) Bring the vol. of fluid in each T.T. up to 2 c.c. with 0-85 S.S.S. (5) Add 2 c.c. casein sol. to each T.T. (6) Set up a similar series of small T.T. containing normal serum, as control. (7) Keep each series of T.T. 30 min. at 50°C. (8) Add to each T.T. 4 drops ascitic acid sol. (9) Determine the complete digesting dose of trypsin. (10) Calculate the amount of trypsin neutralized by the test serum by comparison of the complete digesting dose in this series with that in the normal serum series and with the dose obtained in the absence of any serum.

**Notes.**—<sup>1</sup> Used in the diagnosis of carcinoma and suppurative processes.

**B9-122 GELATIN LIQUEFACTION METHOD: B9-121 GELATIN.** (1) Prepare 10 per cent gelatin in D.W. (2) Add 15 c.c. sensitive litmus sol. **S7-783** per litre. (3) Neutralize exactly. (4) Distribute into T.T. in quantities of 2 c.c. (5) Sterilize 10 min. at 110°C. (6) Keep<sup>1</sup> with precautions against drying.

**Notes.**—<sup>1</sup> Such a gelatin ought, after liquefaction at 40°C., to solidify in less than 2 min. when kept in melting ice.

**B9-122 TRYPSIN STANDARDIZATION.** (1) Take as unit of trypsin a sol. such that 0-1 c.c. liquefies 2 c.c. gelatin sol. in 1 hr. at 41°C. but does not completely liquefy in 45 min.

**B9·123 TEST.** (1) Use serum which has been 24 hr. in contact with clot. (2) Keep serum and trypsin mixture 30 min. at 20C. (3) Make the digest mixture<sup>1</sup> in the water bath at 40C. (4) Keep 1 hr. at 41C.

**Notes.**—<sup>1</sup>Serum, trypsin, and gelatin sol. Use quantities of pure serum ranging from 0·0005 c.c. to 0·4 c.c. Determine the smallest quantity of serum capable of causing a definite retardation in the time of liquefaction of gelatin.

**B9·13 GELATIN LIQUEFACTION METHOD: B9·131 GELATIN.**—(1) Prepare 15 per cent. gelatin in D.W.

**B9·132 TRYPSIN STANDARDIZATION.** (1) Set up mixtures of graded quantities of 1 per cent trypsin suspension with 1 c.c. 15 per cent gelatin. (2) Keep 30 min. at 37C., all the reagents being already at 37C. before mixing. (3) Keep 30 min. at 10C. (4) Take as unit the smallest amount of trypsin causing complete liquefaction.

**B9·133 TEST.**—(1) Set up mixtures of graded quantities of 1·5 test serum with 1 unit of trypsin. (2) Keep 30 min. at 37C. (3) Add 1 c.c. gelatin sol. at 37C. (4) Keep 30 min. at 37C. (5) Keep 30 min. at 10C. (6) Determine the titre of the serum as the smallest amount of serum completely inhibiting the liquefying action of the unit of trypsin.

**B9·14 CALCIFIED MILK METHOD: B9·141 MILK.**—(1) Use milk to which has been added 1·10th its vol. of 10 per cent crystalline calc. chloride sol.

**B9·142 TEST.**—(1) Make a suitable<sup>1</sup> series of dilutions of the trypsin sol. (2) Set out upon a porcelain slab a series of unit vol. of the trypsin dilutions. (3) Add to each of these vol. one unit vol. of 1·8 test, or control serum. (4) Add to each of the mixtures one unit vol. of calcified milk. (5) Mix thoroughly the components of each of the mixtures. (6) Take up a sufficient sample of each mixture into a capillary pipette, separating off the samples from one another, by means of air bubbles. (7) Seal the extremity of the pipette. (8) Keep 20 min. at 50C. in a water bath. (9) Blow out the mixtures into water to determine the occurrence of coagulation or its inhibition.

**Notes.**—<sup>1</sup>A preliminary trial of the potency of the trypsin sol. with regard to the calcified milk must be carried out in order to determine what are suitable dilutions to use.

**B9·2 ANTIVENIN ACTION: B9·21 SUBCUTANEOUS TEST: B9·211.**—(1) Prepare 1·1000 cobra venom in 0·85 S.S.S. (2) Prepare 1·1000 cobra venom 1: anti-serum 1. (3) Keep 15 min. at R.T.

- (4) Inoculate subcutaneously a 1000-grm. rabbit with the mixture.
- (5) Keep the animal under observation for 24 hr.<sup>1</sup>

**Notes.**—<sup>1</sup>If the rabbit survives it is judged that the anti-serum is capable of neutralizing at least 1 minus 0.5–0.5 mgm. cobra venom. The deduction of 0.5 mgm. represents the M.L.D. for a 1000-grm. rabbit.

**B9:212.**—(1) Inject a 2-kilogram. rabbit with 2 c.c. anti-cobra serum subcutaneously. (2) Inject 2 hr. later 1 mgm. cobra venom subcutaneously in this rabbit. (3) Observe<sup>1</sup> the animal.

**Notes.**—<sup>1</sup>If the serum is potent no symptoms should result.

**B9:22 INTRAVENOUS TEST: B9:221.**—(1) Inject a 2-kilogram. rabbit in one ear vein with 2 c.c. anti-cobra serum. (2) Inject 5 min. later into the other vein 1 mgm. of cobra venom. (3) Inject a control rabbit also intravenously with 1 mgm. cobra venom. (4) Observe<sup>1</sup> the animals.

**Notes.**—<sup>1</sup>The control rabbit should die in less than 30 min. If the serum is potent no symptoms should result in the control animal. More exact titration of the serum is effected by first finding the M.L.D. of the venom and then finding the amount of serum which will protect a rabbit against this dose. One anti-venom unit is said to be contained in 1 c.c. of anti-serum which protects against 1 grm. of rabbit.

**B9:222.**—(1) Determine the smallest amount of dry venom which is certainly lethal by intravenous injection in 15 to 20 min. for a 2000-grm. rabbit. (2) Inject intravenously a series of 2000-grm. rabbits with 0.5, 1, 2, 3 c.c., etc., of the test antivenin. (3) Inject 15 min. later into the other ear marginal vein the minimum lethal dose of venom. (4) Calculate<sup>1</sup> the potency of the serum.

**Notes.**—<sup>1</sup>If 1 c.c. of the serum suffices to protect a 2000-grm. rabbit the serum is said to contain 2000 units per c.c.

**B9:3 BACTERICID ALACTION: B9:31. COLONY COUNT METHOD: B9:311.**—(1) Use a strictly sterile technique controlled by culture of reagents throughout the operations. (2) Set up a first series of 9 sterile T.T. containing 1 c.c. dilutions 1-100, 200, 400, 800, 1,600, 3,200, 6,400, 12,800, 256,000<sup>1</sup> inactivated<sup>2</sup> test serum. (3) Set up a second series of 8 T.T. containing Nos. 1 to 4, 1 c.c. 1-100, 200, 400, 800 inactivated normal serum and Nos. 5 to 8,<sup>3</sup> 1, 0.5, 0.2 and 0.1 c.c. 1-10 fresh G. P. serum. (4) Add to the T.T. of the first series and to Nos. 1-4 T.T. of the second series 0.5 c.c. 1-500 24-hr. bouillon culture of bacterial antigen and 0.5 c.c. 1-10 fresh G. P. serum. (5) Add to Nos. 5-8 T.T. of the second series 0.5 c.c. 1-500 24-hr. bouillon culture of bacterial antigen and bring the total vol. of each T.T. up to 2 c.c.

with bouillon. (6) Mix 0.5 c.c. 1-500 24-hr. bouillon culture of bacterial antigen with 7 c.c. melted nutrient agar at 42°C. and plate.<sup>4</sup> (7) Keep all the T.T. mixtures 3 hr. at 37°C. (8) Add each T.T. mixture to 7 c.c. melted nutrient agar at 42°C. and plate. (9) Incubate all the plates made. (10) Count the colonies which develop on each plate after 24, 48 and 72 hr. (11) Compare the results obtained with each other and each with the result of the count giving the original content in bacteria of the bouillon culture used.

**Notes.**—<sup>1</sup>Or other suitable dilutions. <sup>2</sup>30 mm. at 55°C. <sup>3</sup>To determine the bactericidal effect if any of the fresh G.P. complementing serum. <sup>4</sup>To obtain a figure for the original content in bacteria of the 24-hr. bouillon culture of bacterial antigen.

**B9.32 COMPLETE STERILIZATION METHOD: B9.321 ANTI-GEN.**—(1) Use a strictly sterile technique. (2) Set up 10 small T.T. containing 250 c.mm. 0.85 S.S.S. (3) Withdraw 5, 2.5, 50, 25, 50, 25, 50, 25, 50, 25 c.mm. from Nos. 1 to 10 T.T. respectively. (4) Transfer 5 c.mm. of bacterial antigen suspension from a young culture to No. 1 T.T. and 2.5 c.mm. to No. 2 T.T. and mix at the time of addition, thus making 1-50 and 1-100 dilutions of antigen suspension. (5) Sterilize the diluting pipette by aspirating and ejecting boiling water. (6) Allow to cool. (7) Transfer, in the order<sup>1</sup> given, 50 c.mm. of mixture from No. 2 T.T. to No. 3 T.T. and 25 c.mm. from No. 1 T.T. to No. 4 T.T., and mix at the time of addition thus making 1-500 and 1-1,000 dilutions of antigen. (8) Resterilize the pipette and allow to cool. (9) Transfer<sup>2</sup> similarly 50 and 25 c.mm. of the 1-1,000 dilution of antigen to T.T. Nos. 5 and 6, thus making 1-5,000 and 1-10,000 dilutions. (10) Transfer 50 and 25 c.mm. from the 1-10,000 dilution of antigen to T.T. Nos. 7 and 8 thus making 1-50,000 and 1-100,000 dilutions. (11) Transfer 50 and 25 c.mm. from the 1-100,000 dilution of antigen to T.T. 9 and 10 thus making 1-500,000 and 1-1,000,000 dilutions.

**Notes.**—<sup>1</sup>The order is designed to admit of transference from the higher dilution before proceeding to the lower dilution. <sup>2</sup>The pipette is resterilized before being used for the succeeding set of transfers.

**B9.322 ANTIGEN SUSPENSION STANDARDIZATION.**—(1) Fill into a pipette 10 c.mm. 1-1,000,000 antigen suspension. (2) Sow this amount carefully over an agar slope. (3) Repeat the procedure on two other agar slopes. (4) Sterilize the pipette. (5) Sow in similar fashion 10 c.mm. 1-100,000 antigen suspension on each of 3 agar slopes. (6) Incubate. (7) Count the number of colonies which develop.

(8) Calculate the number of bacteria per c.c. contained in the original suspension.

**Notes.**—<sup>1</sup>The dilution used will depend entirely on the concentration of the suspension used, the object being to obtain sufficiently isolated colonies for counting.

**B9 323 TEST.**—(1) Take a sterile capillary stemmed pipette fitted with rubber teat and place a mark on its capillary stem about 2 cm. from the lower end. (2) Draw up mannite litmus bouillon (M4:552 into the bulb of the pipette. (3) Draw up one unit vol. of test serum. (4) Admit an air bubble to separate the mannite litmus bouillon from the following serum antigen mixture. (5) Draw up one unit vol. of antigen dilution. (6) Mix together serum and antigen without driving the sterile bouillon down sufficiently far from the barrel of the pipette to contaminate it. (7) Draw up the mixed serum and antigen dilution into the middle region of the capillary stem. (8) Seal the lower end of the pipette and replace the rubber teat with a sterile wool plug. (9) Continue similarly with fresh pipettes for each of the dilutions of antigen suspension proceeding always from the greater dilution to the less. (10) Keep the sealed pipette 20 hr. at 37°C. (11) Draw out, without heating the serum antigen mixture, the extreme lower end of the capillary stem into a fine hair capillary, and seal once more. (12) Establish a condition of negative pressure in the interior of the pipette by fitting over its upper end a collapsed rubber teat. (13) Regulate this negative pressure carefully by means of the finger and thumb applied to the teat. (14) Break the fine hair capillary, and so allow the serum antigen mixture to ascend into the barrel and mix with the bouillon. (15) Seal the lower end of the capillary stem again. (16) Keep the pipettes thus treated 24 hr. at 37°C. (17) Examine for growth in, and fermentation of, the mannite bouillon. (18) Determine on which dilution of bacterial antigen the test serum has acted with complete bactericidal effect. (19) Set up a control on similar lines with normal serum. (20) Calculate the complete bactericidal point.<sup>1</sup>

**Notes.**—<sup>1</sup>The data which are available are the number of organisms contained in the antigen suspension, and the highest dilution in which these have been completely killed off by the test serum. The complete bactericidal point is given by the number of bacteria which 1 c.c. of the test serum is capable of killing off.

#### **B9:4 BACTERIOLYTIC ACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup>V. also B9:63.

**B9:41: B9:411 ANTIGEN UNDER TEST.** (1) Set up 5 T.T. Nos. 1 to 5 containing 2 c.c. of dilutions of bacteriolytic serum in bouillon

representing 2, 4 and 8 times as much serum as is contained in 2 c.c. of the titre dilution, No. 4 containing 2 c.c. 1-100 normal serum, and No. 5 containing 2 c.c. bouillon only. (2) Suspend in each T.T. 2-mgm. loopfuls of an 18 to 24-hr. culture of test bacterial antigen. (3) Inject 1 c.c. of each mixture intraperitoneally in each of 250-grm. G.P. (4) Remove samples of peritoneal exudate immediately after injection, and at intervals of 10, 20, 30, 40 and 60 min. after injection. (5) Examine<sup>2</sup> in hanging drop and stained film preparation. (6) Note symptoms and time of death if it occurs.

**Notes.**—<sup>1</sup>Chiefly used for test of *V. cholerae*. <sup>2</sup>If the samples from G. P. Nos. 1, 2, and 3 show spherulation and agglutination of bacteria within the 60 min. interval, while those from G.P. Nos. 4 and 5 show motile unclumped organisms for the same interval, the test may be taken as positive.

**B9-412 ANTIGEN UNDER TEST.**—(1) Make a first dilution of 1-100 inactivated bacteriolytic serum in bouillon. (2) Make further dilutions from this first dilution. (3) Mix 2 c.c. of each of the dilutions made with 2 mgm. loopfuls<sup>1</sup> of test culture. (4) Inject 1 c.c. of each suspension thus made intraperitoneally in 250-grm. G.P. (5) Examine samples of peritoneal exudate at intervals. (6) Observe the effect<sup>2</sup> of the injection upon the G.P. (7) Make control injections.

**Notes.**—<sup>1</sup>A 4-mm. loop holds 2 mgm. of bacterial culture. <sup>2</sup>Symptoms death or recovery.

**B9-413 SERUM<sup>1</sup> UNDER TEST.**—(1) Collect the test serum with sterile precautions. (2) Inactivate by heating 30 min. at 56°C. (3) Set up 5 T.T. containing Nos. 1 to 4, 2 c.c. of dilutions<sup>2</sup> 1-20, 100, 200, 400 in bouillon of test serum, and No. 5, 2 c.c. bouillon only. (4) Make a suspension of 2 mgm. of an 18 to 24-hr. culture of known<sup>3</sup> bacterial antigen in each of the 5 T.T. (5) Inject 1 c.c. of the suspensions so made intraperitoneally in 250-grm. G.P. (6) Make a suspension of 2 mgm. of the bacterial antigen culture in 2 c.c. 1-10 normal serum. (7) Inject 1 c.c. of this suspension also intraperitoneally in a 250-grm. G.P. (8) Shave a small area on the abdomen of the G.P. (9) Anæsthetize the animals lightly. (10) Make a small incision through the skin of the abdomen. (11) Pass a capillary pipette, the barrel of which is kept closed with the index finger, quickly into the abdomen. (12) Remove the index finger and move the tube gently about. (13) Withdraw the pipette with a sudden movement. (14) Carry out this operation immediately after injection and 10, 20, 30, 40 and 60 min. after injection. (15) Make hanging drop and film preparations with the exudate obtained.

(16) Examine each preparation for loss of motility, agglutination, and disintegration in the antigenic bacteria. (17) Observe<sup>4</sup> the effect of the injections upon the G.P.

**Notes.**—<sup>1</sup>As for the diagnosis of disease. <sup>2</sup>Or other dilutions which may be preferable. <sup>3</sup>The test has been specially employed in the case of *V. cholera*. <sup>4</sup>Symptom: death or recovery. The control G.P. should die and some at least of the test serum G.P. should live.

**B9-42 V. CHOLERÆ: B9-421.**<sup>1</sup>—(1) Shave the abdomens of a series of young 200-grm. G.P. (2) Make a small incision in the skin. (3) Inject with a blunt canula intraperitoneally mixtures of 1 c.c. 1-10, 4,000, 10,000 anti-serum with 2 mgm. of an 18-hr. virulent culture of *V. cholera* suspended in 1 c.c. bouillon. (4) Examine samples of the aspirated peritoneal exudate after 10, 20, 30 min. (5) Examine the animal after 24 hr. and calculate the titre<sup>1</sup> of the anti-serum.

**Notes.**—<sup>1</sup>The unit of bacteriolytic serum is the smallest quantity of serum which suffices to cause complete bacteriolysis of 2 mgm. of a normal cholera virus within 1 hr., and is able to protect the animal from death for 24 hr. A 'normal' virus is one which is fatal to a 200-grm. G.P. by intraperitoneal injection in a dose of 0.1 to 0.2 mgm. The potency of the serum may be expressed as units per c.c. Control G.P. which receive culture only, or culture in mixture with normal serum, should show numerous living motile unagglutinated organisms in the sample of mixture examined after 60 min. and must die within 24 hr. The test is used with appropriate modifications for the diagnosis of the disease with patient's serum, and for identification of the organism with a known antiserum.

**B9-5 COBRA VENOM REACTION: B9-51 PSYCHO-REACTION: B9-511.**<sup>1</sup>—(1) Prepare a stock sol. of cobra venom: dried venom 0.2: D.W. 10: glycerin 10. (2) Keep in the ice chest. (3) Dilute for use 1-50 with 0.85 S.S.S. (4) Set up 8 small T.T. containing 0.35, 0.35, 0.35, 0, 0, 0, 0, c.c. inactivated<sup>2</sup> test serum and 1, 0.5, 0.25, 0.1 1.0, 0.5, 0.25 and 0.1 c.c. diluted cobra venom sol. (5) Add 0.5 c.c. 10 per cent suspension washed erythrocytes. (6) Make up the vol. in each T.T. with 0.85 S.S.S. to 1.85 c.c. (7) Shake gently to mix. (8) Keep 2 hr. at 37C. (9) Keep 20 to 24 hr. in the ice chest. (10) Shake up the T.T. to mix the contents. (11) Read the results.

**Notes.**—<sup>1</sup>In many psychoses, e.g. depressive manic and depressive psychosis, the serum of the patient has the power of neutralizing the haemolytic power of cobra venom. <sup>2</sup>30 min. at 56C.

**B9-52 SYPHILIS: B9-521.** (1) <sup>1</sup>Standardize desiccated venom against known normal and known syphilitic sera. (2) Set up 4 small T.T. containing 1 c.c. 1 per cent suspension of washed human erythrocytes and 1 c.c. 1-10,000, 1-20,000, 1-30,000, 1-40,000 cobra venom in 0.85

S.S.S. (3) Keep 1 hr. at 37C. (4) Shake to mix. (5) Keep in the ice chest overnight. (6) Shake to mix. (7) Read<sup>2</sup> the results 1hr. later.

**Notes.**—<sup>1</sup>In syphilis the resistance of the erythrocytes to hæmolysis are increased. <sup>2</sup>There are 3 readings to be made :—the time of commencement of hæmolysis, the degree of completeness of hæmolysis, and the degree to which the erythrocytes sink to the bottom of the T.T.

**B9 522.**<sup>1</sup>—(1) Prepare an alcoholic extract :—serum 1 ; methyl alc. 7. (2) Keep 15 min. at 37C. (3) Centrifugalize. (4) Pipette off the clear S.N.F. for use. (5) Set up a series of small T.T. each containing 5 vol. 5 per cent washed sheep erythrocyte suspension and 5 vol. 0.01 per cent cobra venom in 0.85 S.S.S. (6) Add to No. 1 T.T. 1 vol. alcoholic extract of known normal serum to No. 2 T.T. 1 vol. alcoholic extract of known positive syphilitic serum, and to the remainder 1 vol. alcoholic extract of the various test sera. (7) Shake gently to mix. (8) Keep at 37C. and watch the control known normal serum T.T. for hæmolysis.<sup>2</sup> (9) Remove the T.T. as soon as this occurs. (10) Read the results.

**Notes.**—<sup>1</sup>The test is dependent on the fact that lecithin in conjunction with cobra venom is capable of producing hæmolysis of washed erythrocytes on a species of animal whose erythrocytes are not affected by cobra venom alone. <sup>2</sup>Test tubes containing normal serum show hæmolysis after about 15 min.

**B9 53 CANCER : B9 531.**<sup>1</sup>—(1) Set up 2 rows of 4 small T.T. containing in the front row 0.1, 0.2, 0.3, and 0.5 c.c. inactivated<sup>2</sup> test serum and the same amount in the back row. (2) Make up the vol. of fluid in each T.T. to 0.5 c.c. with 0.85 S.S.S. (3) Add to each T.T. of the front row 0.3 c.c. 1-5,000 cobra venom and to each T.T. of the back row 0.15 c.c. 1-5,000 cobra venom. (4) Add to each T.T. of both rows 5 drops 10 per cent suspension horse erythrocytes. (5) Shake gently to mix. (6) Keep at 37C. (7) Read the results<sup>3</sup> after 15, 30, 60, 120, 180 min.

**Notes.**—<sup>1</sup>About 70 per cent of cancer sera are said to react positively. It is invariably strongly positive in pregnancy, beginning with the 4th month and disappearing about 3 weeks after parturition. Positive reactions may also be found in cases of icterus and of advanced tuberculosis. 230 min. at 56C. <sup>2</sup>Degree of neutralization of the hæmolytic power of the cobra venom.

**B9 6 COMPLEMENT ACTION : B9 61 COMPLEMENT SPLITTING.**—(1) Mix 0.5 c.c. fresh G.P. serum with 4.1 c.c. N-250 or N-300 hydrochloric acid in D.W. (2) Leave 1 hr. at R.T. (3) Centrifuge. (4) Make a suspension of the sediment in a suitable quantity of D.W. = 'middle piece.' (5) Filter the S.N.F. (6) Add to the filtrate 0.4 c.c.

10 per cent sod. chloride which contains N-25 or N-30 sod. hydroxide to neutralize and make isotonic=<sup>1</sup> end piece.<sup>2</sup>

**B9·62 HÆMOLYTIC COMPLEMENT.**<sup>1</sup>—(1) Set up a series of T.T. containing diminishing amount of fresh<sup>2</sup> complementing serum with a constant and sufficient dose of inactivated hæmolytic serum, and a fixed dose of erythrocyte antigen. (2) Keep 1 hr. at 37C. (3) Note the T.T. containing the least amount of complement showing complete hæmolysis—minimal hæmolytic dose of complement, or one unit of complement. (4) Indicate the potency of the complementing serum as the amount in c.c. which contains one minimal hæmolytic dose.

**Notes.**—<sup>1</sup>V. also **B9·7** and **B10·8**. <sup>2</sup>Collected a few hr. before use from two or more fasting G.P.

**B9·63 BACTERIOLYTIC COMPLEMENT.**<sup>1</sup>—(1) Set up, with strictly sterile precautions, a series of T.T. containing diminishing amount of fresh<sup>2</sup> complementing serum, with a constant and sufficient dose of inactivated bacteriolytic serum, and a fixed dose of bacterial antigen. (2) Keep 90 min. at 37C. (3) Prepare and stain films and prepare hanging drops of the suspensions. (4) Note<sup>3</sup> the changes in the organisms shown in these films and hanging drops<sup>4</sup> as compared with films and hanging drops made from the bacterial antigen suspension which has simply been kept 90 min. at 37C.

**Notes.**—<sup>1</sup>V. also **B9·5**. <sup>2</sup>Collected a few hr. before use from two or more fasting G.P. <sup>3</sup>For quantitative purposes a colony count method **B9·411** is necessary, where definite quantities of the mixtures are sown on plates. <sup>4</sup>The changes shown are loss of motility, spherulation, agglomeration, and disintegration of the bacteria.

### **B9·7 COMPLEMENT FIXATION ACTION.**<sup>1</sup>

**Notes.**—<sup>1</sup>V. also Wassermann reaction **B10·8**.

### **B9·71 GENERAL TEST.**<sup>1</sup>

**Notes.** <sup>1</sup>Applicable for the most part, with suitable modification, to all complement fixation tests.

**B9·711 ERYTHROCYTES.**<sup>1</sup>—(1) Aspirate blood from the jugular vein of the sheep with a sterile syringe. (2) De fibrinate<sup>2</sup> (**A3·412**, **B9·724**). (3) Strain through a double layer of fine sterilized gauze into centrifuge tubes. (4) Add to each tube about 4 times the amount of 0·85 S.S.S. as of defibrinated blood. (5) Centrifugalize. (6) Remove the S.N.F. (7) Replace it by fresh 0·85 S.S.S. (8) Centrifugalize. (9) Wash in this way three times<sup>3</sup> with 0·85 S.S.S. (10) Suspend the washed erythrocyte sediment in an equal quantity of 0·85 S.S.S. = 0 per cent stock

suspension. (11) Keep<sup>1</sup> in the ice chest until required for use. (12) Dilute 1-10 for use with 0.85 S.S.S.

**Notes.** <sup>1</sup>F. also **A3-411** to **3-413**, **A3-622**. <sup>2</sup>Instead of defibrination, citration may be employed. <sup>3</sup>The first washing may be done the day before the test. <sup>4</sup>Other methods of preservation are given under **B9-721** to **B9-726**. The erythrocytes may be sensitized (**B10-914**) before use in test.

**B9-712 HÆMOLYTIC SERUM.**—(1) Select not less than 6<sup>1</sup> large healthy rabbits. (2) Inject each intraperitoneally with a first dose of 2.5 c.c. 50 per cent stock suspension of erythrocytes. (3) Give a further injection every 5d., each time increasing the dose until after six injections it has reached 10 c.c. (4) Repeat this dose twice. (5) Aspirate 5 c.c. blood from the heart. (6) Inactivate the serum obtained by keeping it 30 min. at 56°C. (7) Estimate by trial the titre of the serum (**B9-713**). (8) Aspirate 25 c.c. blood from the heart of such rabbits as give the desired titre<sup>2</sup> (**B9-7131**). (9) Keep the rabbits for re-injection. (10) Store the serum<sup>3</sup> obtained in glass capsules. (11) Keep in the ice chest until required for use.

**Notes.**—<sup>1</sup>Deaths from anaphylactic shock are apt to take place in the process of immunization and hence the necessity of using as many as six to begin with. <sup>2</sup>0.0005 or higher. <sup>3</sup>If there is doubt about the asepticity of the operations, add 1 vol. of a mixture, glycerin 95, phenol 5, to 9 vol. of the serum before measuring it into the capsules.

**B9-713 HÆMOLYTIC SERUM STANDARDIZATION.**—(1) Make a series of dilutions<sup>1</sup> of serum 1-400, 600, 800, 1,000, 1,500; 2,000, 3,000, 4,000 and 5,000, corresponding to dilutions of 0.0025, 0.0016, 0.0012, 0.001, 0.00066, 0.0005, 0.00033, 0.00025 and 0.0002 parts of serum in 1 part of diluent. (2) Set out 12 small T.T. (3) Add to Nos. 1 to 9 T.T. 1 vol. of each of the serum dilutions in order. (4) Add to No. 10 T.T. 1 vol. of 1-400 serum dilution. (5) Add 2 vol. 0.85 S.S.S. to Nos. 1 to 9 T.T., 3 vol. to Nos. 10 and 11, and 4 vol. to No. 12 T.T. (6) Add 1 vol. of 1-2 fresh G.P. complementing serum to Nos. 1 to 9 T.T., and to No. 11 T.T. (7) Add to all the T.T. 1 vol. 5 per cent erythrocyte suspension made from the 50 per cent stock suspension. (8) Shake gently to mix. (9) Keep 30 min.<sup>2</sup> at 37°C. (10) Determine the minimum hæmolytic dose (M.H.D.).<sup>3</sup> (11) Dilute the serum appropriately according to the determination. (12) Transfer to sterile<sup>4</sup> glass capsules and seal. (13) Heat 30 min. at 56°C. on three successive days.<sup>4</sup>

**Notes.**—<sup>1</sup>Either of these figures, which are equivalent, may be used to indicate the titre of the serum. <sup>2</sup>The time interval used varies between 30 min. and 2 hrs. <sup>3</sup>The titre of the serum is given by the amount of serum (M.H.D.) present in the last T.T. of

the series Nos. 1 to 9, in which sol. of all the erythrocytes is complete. A serum with a titre value between 0.0002 and 0.0005 is quite satisfactory. The dose for the test is 2 minimum hæmolytic doses, e.g., if the titre value is 0.0001, the amount of serum representing 2 minimum hæmolytic doses will be 0.0008 c.c. **¶** Also. **B10-813.** **¶**If all the manipulations have been carried out with sterile precautions this sterilization is unnecessary. The serum may also be preserved in the undiluted state.

**B9-714 COMPLEMENT.**—(1) Anaesthetize<sup>1</sup> a non-pregnant G.P. on the day of the test. (2) Suspend the animal head downwards. (3) Sever the arteries and veins on one side of the neck.<sup>2</sup> (4) Collect all the blood in centrifuge tubes. (5) Centrifugalize immediately before coagulation<sup>3</sup> takes place. (6) Pipette off the serum. (7) Keep in the ice chest till required for use. (8) Use the serum fresh.

**Notes.** <sup>1</sup>Or knock the head of the G.P. slightly on the edge of the table merely to stun it. <sup>2</sup>Blood may be obtained from the heart, if desired, and thus the animal retained for further use. Blood in sufficient quantity may also be obtained from the ear. <sup>3</sup>It is often more satisfactory to allow spontaneous coagulation to take place and to use the separated serum. Or again the blood may be defibrinated (**A3-412**) with a piece of cotton wool on the end of an iron wire, and then centrifugalized.

**B9-715 COMPLEMENT STANDARDIZATION.**<sup>1</sup>—(1) Set up 12 small T.T. (2) Add to Nos. 1 to 9, 1 c.c. of dilution of hæmolytic serum containing 2 M.H.D. (**B9-8133**). (3) Add to No. 10 T.T. 1 c.c. dilution of hæmolytic serum containing 1 minimum hæmolytic dose.<sup>2</sup> (4) Add to each T.T. in serial order 0.3, 0.1, 0.15, 0.5, 0.55, 0.6, 0.65, 0.7, 0.8, 1, 1, and 0 c.c. 1-20 fresh G. P. complementing serum. (5) Make up the vol. in each T.T. to 4 c.c. with 0.85 S.S.S. (6) Add 1 c.c. erythrocyte suspension to each T.T. (7) Shake gently to mix. (8) Keep 30 min.<sup>3</sup> at 37°C. (9) Determine<sup>4</sup> the minimum hæmolytic dose (M.H.D.).

**Notes.**—<sup>1</sup>It may be advisable to standardize the complement in presence of the antigen to be used in the test (**B10-916**). <sup>2</sup>No. 10 T.T. controls the standardization of hæmolytic serum (**B9-713**) with excess of complementing serum. In this T.T. hæmolysis should be complete. The first T.T. in the series in which complete hæmolysis occurs contains one minimum hæmolytic dose of complementing serum. Nos. 11 and 12 T.T. are T.T. controlling the absence of hæmolysis by complementing serum without the addition of hæmolytic serum, and by simple sol. <sup>3</sup>The time interval varies in different methods between 30 min. and 2 hr. and also according as an incubator or water bath is used. <sup>4</sup>The M.H.D. is given by the amount of serum present in the first T.T. of the series Nos. 1 to 9 in which sol. of all the erythrocytes is complete. It is the least amount of complementing serum capable of bringing about complete hæmolysis.

**B9-716 ANTIGEN STANDARDIZATION.**<sup>1</sup>—(1) Set up 24 small T.T. in 2 rows of 12 T.T. each. (2) Add to Nos. 1 to 11 T.T. of each row, pair and pair, 0.2, 0.16, 0.14, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005 and

0.004 c.c. dilution of antigen. (3) Bring the vol. in all 24 T.T. to 2 c.c. with 0.85 S.S.S. (4) Add to each T.T. of No. 1 row 0.2 c.c. inactivated<sup>2</sup> known anti-serum<sup>3</sup> and to each T.T. of No. 2 row 0.2 c.c. inactivated<sup>2</sup> known normal serum.<sup>4</sup> (5) Add to each T.T. 2 M.H.D.<sup>5</sup> complementing serum (**B9.715**) contained in 1 c.c. 0.85 S.S.S. (6) Shake gently to mix. (7) Keep 60 min. at 37°C. (8) Add to each T.T. 2 M.H.D. (**B9.713**) inactivated<sup>2</sup> hæmolytic serum, and 1 c.c. 5 per cent erythrocyte suspension. (9) Keep T.T. 30 min. at 37°C. (10) Keep 18 hr.<sup>6</sup> at a temperature not exceeding 15°C. (11) Note the minimum amount of antigen in No. 1 row which gives complete fixation of complement with the known anti-serum, and the maximum amount of antigen in No. 2 row which fails to prevent the occurrence of complete hæmolysis. (12) Select<sup>7</sup> as antigen unit an amount of antigen between these two values. (13) Place two units of antigen diluted to 3 c.c. with 0.85 S.S.S. in a T.T., and 1 c.c. 5 per cent erythrocyte suspension. (14) Keep 60 min. at 37°C. (15) Reject the antigen as unsuitable for test if any hæmolysis appears.

**Notes.**—<sup>1</sup>In this standardization are included the antigenic power and the anti-complementary power. <sup>2</sup>30 min. at 56°C. <sup>3</sup>Or, as it will be described under the Wassermann reaction, etc., 'known positive serum.' <sup>4</sup>Or, as it is sometimes described, 'known negative serum.' <sup>5</sup>In some methods only 1 M.H.D. is used. <sup>6</sup>In some methods of standardization of antigen, and also in the test itself, the reading is simply taken after 1 or 2 hr. at 37°C., or again without specifying the time interval after the occurrence of complete hæmolysis in the special control tubes. <sup>7</sup>The unit for the test should be a multiple of the antigenic unit as given in No. 1 row of T.T. but should not exceed one-half the anti-complementary unit as given in No. 2 row of T.T.

**B9.717 TEST.**—(1) Set up in two rows, pairs of small T.T., a front one and a back one for each inactivated<sup>1</sup> test serum, and for the inactivated known anti-serum<sup>2</sup> and the inactivated known normal<sup>3</sup> serum, and in addition one T.T. each for the antigen control, the hæmolytic serum control, and the erythrocyte suspension control. (2) Add to each front row T.T. one unit of antigen contained in 1 c.c. 0.85 S.S.S. and to the antigen control T.T. 2 units contained in 2 c.c. 0.85 S.S.S. (3) Add to each of the pair of T.T. corresponding to a given serum 0.2 c.c. of that serum to the front row T.T. and 0.4 c.c. of the serum to the back row T.T. (4) Bring the vol. of fluid in all T.T. up to 2 c.c. with 0.85 S.S.S. (5) Add to all the T.T., except the erythrocyte suspension control T.T., 2 M.H.D. (**B9.715**) complementing serum contained in 1 c.c. 0.85 S.S.S. (6) Shake gently to mix. (7) Keep 60 min. at 37°C. (8) Add to each T.T. 2 M.H.D. (**B9.713**) inactivated<sup>1</sup> hæmolytic serum and 1 c.c. 5 per cent erythrocyte suspension.<sup>4</sup> (9) Shake gently to mix.

(10) Keep 30 min. at 37°C. (11) Keep 18 hr.<sup>5</sup> at a temperature not exceeding 15°C. (12) Examine the control T.T. (13) Examine the T.T. containing known anti-serum and normal sera. (14) Examine the test sera T.T. (15) Record the result.<sup>6</sup>

**Notes.**—<sup>1</sup>30 min. at 56°C. <sup>2</sup>Or, as it will be described under the Wassermann reaction, etc., 'known positive serum.' <sup>3</sup>Or, as it is sometimes described, 'known negative serum.' <sup>4</sup>The erythrocytes of the suspension may be sensitized with the hemolytic serum as a preliminary to addition. <sup>5</sup>The reading may be simply taken after 1 or 2 hr. at 37°C. or again, without specifying the time interval, after the occurrence of complete hemolysis in the special control T.T. <sup>6</sup>Absence of hemolysis in positive cases.

## B9-72 PRESERVATION OF REAGENTS.<sup>1</sup>

**Notes.**—<sup>1</sup>For use in complement fixation reactions.

**B9-721 ERYTHROCYTES.**<sup>1</sup>—(1) Prepare washed<sup>2</sup> deposit sheep erythrocytes 5 : 10 per cent formalin 0.1. (2) Prepare at time of use with a 1 c.c. pipette previously wetted with 0.85 S.S.S. : formalinized suspension 6 : 0.85 S.S.S. 100.

**Notes.**—<sup>1</sup>Formalinized erythrocytes keep for at least a week. <sup>2</sup>The blood is collected at the slaughter house as aseptically as possible in sterile bottles containing 1.5th of their vol. of 5 per cent citrated 0.85 S.S.S. As soon as possible after collection the erythrocytes are washed. The final deposit is packed as firmly as possible by prolonged centrifuging.

**B9-722 ERYTHROCYTES.**—(1) Collect blood under strict aseptic conditions by venepuncture. (2) Draw up into a sterile 20 c.c. syringe 10 c.c. 1.5 per cent citrated 0.85 S.S.S. (3) Follow on by aspiration of 10 c.c. sheep's blood. (4) Empty the contents of the syringe into a large sterile T.T. containing 10 c.c. 1.5 per cent citrated 0.85 S.S.S. (5) Keep for use in a cool place. (6) Draw off at time of use with aseptic precautions the quantity required. (7) Wash three times with 0.85 S.S.S. in a centrifuge. (8) Make a 10 per cent suspension in 0.85 S.S.S. for use in test.

**B9-723 ERYTHROCYTES.**—(1) Prepare : formalin 0.5 : 1 per cent ammon. oxalate in 0.85 S.S.S. : sheep blood 100. (2) Wash at time of use.

**B9-724 ERYTHROCYTES.**—(1) Collect the blood at the slaughter house as aseptically as possible into a stoppered glass bottle containing a few pieces of glass and iron wire. (2) Fill only half full. (3) Shake continuously to defibrinate. (4) Keep in the ice chest.<sup>1</sup> (5) Wash<sup>2</sup> at time of use three times with 0.85 S.S.S.

**Notes.**—<sup>1</sup>Will keep thus 3 or 4d. <sup>2</sup>The first washing may be done the day before use.

**B9·725 ERYTHROCYTES.** (1) Wash sheep erythrocytes twice with 0·85 S.S.S. (2) Make up a 5 per cent suspension in 0·85 S.S.S. (3) Prepare 5 per cent suspension erythrocytes 5; 1-10 formalin<sup>1</sup> 0·1. (4) Keep<sup>2</sup> for use.

**Notes.**—<sup>1</sup>The 1-10 formalin should be prepared with 0·85 S.S.S. <sup>2</sup>Will keep 15 d.

**B9·726 ERYTHROCYTES.** (1) Take the blood into a flask containing:—sod. citrate 7·5; formalin 2; 0·85 S.S.S. 500. (2) Keep in the ice chest. (3) Wash the erythrocytes each time as required.

**B9·727 COMPLEMENT.**—(1) Add to each c.c. fresh G.P. serum 0·1 c.c. saturated sod. chloride. (2) Keep<sup>1</sup> at a temperature not exceeding 6C. (3) Dilute at time of use with 3 times its vol. of D.W.

**Notes.**—<sup>1</sup>Will keep, it is said, 2 weeks.

**B9·728 TEST SERUM.**—(1) Prepare<sup>1</sup>:—inactivated test serum 1; glycerin 1.

**Notes.**—<sup>1</sup>The addition of the preservative glycerin has no effect on the Wassermann reaction.

**B9·729 TEST SERUM.** (1) Add 1 in 10 of 5 per cent carbolyzed glycerin.

**B9·730 BACTERIA : B9·731 GENERAL.**<sup>b</sup> (1) Prepare as antigen a rather thick<sup>2</sup> suspension of bacteria from a 24-hr. agar culture in 0·85 S.S.S. (2) Determine the maximum dose of suspension which does not produce inhibition of hæmolysis. (3) Use one half this dose in test in a vol. of 0·5 c.c. (4) Set out a series of T.T. containing dilutions of inactivated<sup>3</sup> serum in quantities of 5 c.c. (5) Add 0·5 c.c. dilution of antigen suspension. (6) Add 1 M.H.D. (B9·715) complement in a vol. of 0·5 c.c. (7) Keep 1 hr. at 37C. (8) Add 0·5 c.c. 5 per cent suspension of sheep erythrocytes and 3 M.H.D. (B9·713) of inactivated<sup>3</sup> anti-sheep hæmolytic serum. (9) Keep 2 hr. at 37C. (10) Read<sup>4</sup> the result. (11) Keep overnight in the ice chest. (12) Reac<sup>4</sup> again.

**Notes.**—<sup>1</sup>Applicable, *e.g.*, to *B. pestis*, *B. anthracis*, streptococcus, gonococcus, meningococcus, *B. typhosus*, *B. coli*, *B. tuberculosis*, *B. pertussis*, *Sp.icterohæmorrhagiæ* and other infections. <sup>2</sup>*e.g.*, 5 c.c. of 0·85 S.S.S. to an agar slope of *B. typhosus*; 1 c.c. 0·85 S.S.S. to 80 mgm. *B. tuberculosis*. <sup>3</sup>30 min. at 56C. <sup>4</sup>The usual controls (**B 9, 71**) should be set up.

**B9·732 GONOCOCCUS.**—(1) Make a thick suspension of a 24-hr. growth of gonococci in 0·85 S.S.S. (2) Enumerate the number of organisms per c.c. of suspension and determine the degree of dilution required to make the content 1000 million per c.c. (3) Add to the suspension a few c.c. N-10 sod. hydroxide which dissolves the gonococci.

(4) Neutralize the suspension to litmus with N-10 hydrochloric acid. (5) Dilute to that vol. which would have made the original suspension into one containing 1000 million organisms per c.c. (6) Use in test as antigen.

**B9-733 GONOCOCCUS.**—(1) Wash off a 24-hr. growth with sterile D.W. (2) Centrifugalize at high speed. (3) Remove the S.N.F. (4) Repeat the process three times using D.W. for the washing. (5) Add 50 c.c. sterile D.W. to each 0.5 gm. of sediment. (6) Keep 30 min. at 56C. (7) Keep 10d. at 37C, shaking for about 10 min. daily. (8) Centrifugalize. (9) Pipette off the S.N.F. for use as antigen with the addition of a drop of carbolic acid or lysol as preservative.

**B9-734 GONOCOCCUS.**—1) Wash off a 48-hr. growth with 0.85 S.S.S. (2) Shake to break up clumps. (3) Keep 1 hr. at 56C. (4) Add 0.1 c.c. 1 per cent phenol per c.c. as preservative. (5) Keep as stock. (6) Dilute 1-10 for use. (7) Test for antigenic potency and for anti-complementary action (B9-716).

**B9-735 MENINGOCOCCUS.**—(1) Make a suspension of strength 0.5 mgm. per c.c. with 0.85 S.S.S. from a culture not more than 24 hr. old. (2) Keep 5 min. at 100C. Use as antigen in test.

**B9-736 B. MALLEI.**—(1) Grow a freshly isolated strain on glycerin agar M3-111, for 36 hr. (2) Sterilize the culture by heating 2 hr. at 60C. (3) Make a suspension of a culture with 10 c.c. carbolized 0.85 S.S.S. (4) Shake in a mechanical shaker 4 d. (5) Centrifuge at high speed. (6) Pipette off the S.N.F. for use.

**B9-737 B. MALLEI.**—(1) Grow the organism on glycerin agar (M3-111) 1.6 per cent acid to phenolphthalein. (2) Make a suspension of about the turbidity of milk in D.W. (3) Sterilize 2 hr. at 60C. (4) Shake to break up clumps 3 hr. on two successive days. (5) Add sufficient sod. chloride to make isotonic and 0.5 per cent phenol to preserve. (6) Keep in a cool dark place. (7) Use as antigen in test.

**B9-738 B. TUBERCULOSIS.**—(1) Prepare a suspension of killed B. tuberculosis. (2) Centrifugalize till the deposit is of constant vol. (3) Use<sup>1</sup> the S.N.F. as antigen for test.

**Notes.**—<sup>1</sup>It will keep in sealed capsules for 5 weeks.

**B9-739 B. TUBERCULOSIS.**—(1) Sow on the surface of 5 per cent glycerin veal bouillon. (2) Incubate 10 weeks. (3) Transfer the growth to a bottle. (4) Dehydrate by testing with abs. alc. which should be repeatedly changed. (5) Replace the alc. with ether. (6) Shake 2 hr.

(7) Centrifugalize at high speed. (8) Treat the sediment with chloroform. (9) Shake 2 hr. (10) Mix with 3 vol. ether. (11) Shake 2 hr. (12) Centrifugalize. (13) Repeat the procedure using ether and chloroform alternately until five extractions have been completed. (14) Dry the sediment *in vacuo* over sulphuric acid or calc. chloride. (15) Keep for use. (16) Grind up 100 mgm. with 3 c.c. 0·85 S.S.S. in a mechanical mill overnight. (17) Draw off the fluid. (18) Wash the flask and balls of the mill with frequent changes of 0·85 S.S.S. (19) Mix the first fluid drawn off with the washings. (20) Bring up the total vol. to 100 c.c. with 0·85 S.S.S. (21) Add 0·5 per cent phenol. (22) Use as antigen in test.

### **B9·74 OTHER<sup>1</sup> INFECTIONS.**

**Notes.**—<sup>1</sup> Other than bacterial.

**B9·741 DOURINE.**—(1) Grind up the spleen of a rat heavily infected with *T. equiperdum* in 30 c.c. 0·85 S.S.S. (2) Filter the suspension. (3) Use in test after standardization.

**B9·742 DOURINE.**—(1) Inoculate intraperitoneally not less than 10 large white rats with 0·3 c.c. of dourine rat blood. (2) Make an examination of the blood of each rat 48 hr. after inoculation. (3) Sort out the rats with heavy and light infections. (4) Make a second blood examination later<sup>1</sup> to determine the time for bleeding. (5) Anæsthetize the rat lightly. (6) Sever the arteries and veins of one side of the neck close to the shoulder without severing the trachea. (7) Collect the blood in 1·5 per cent citrated 0·85 S.S.S.<sup>2</sup> (8) Strain the citrated blood through two layers of sterile gauze into centrifuge tubes of about 10 mm. diameter.<sup>3</sup> (9) Centrifugalize not longer than 5 min.<sup>4</sup> at 1700 revolutions. (10) Draw off the cloudy S.N.F. into fresh tubes. (11) Add 1·5 per cent citrated 0·85 S.S.S. to each of these tubes. (12) Centrifugalize. (13) Draw off and reject as much of the upper portion of S.N.F. as is clear and free from trypanosomes. (14) Collect from each tube the remaining upper pure white layer of trypanosomes. (15) Wash by centrifugalization with 0·85 S.S.S. (16) Add twice the vol. of formalinized glycerin sol.<sup>5</sup> to the sedimented trypanosomes. (17) Keep on ice till required for use. (18) Use as antigen in test.

**Notes.**—<sup>1</sup> The result of the first blood examination will indicate approximately the time for a second examination, and on the latter will be determined the time for bleeding the rats. <sup>2</sup> The vol. of blood should be about equal to that of the citrated salt sol. <sup>3</sup> When wider tubes are used it is more difficult to separate the trypanosomes. <sup>4</sup> So that the bulk of the blood cells are thrown down while the trypanosomes remain in suspension. <sup>5</sup> Formalin 0·1; pure neutral glycerin 10; 0·85 S.S.S. 90.

**B9\*743 ECHINOCOCCUS.**—(1) Obtain hydatid cyst fluid from a sheep cyst. (2) Keep 20 min. at 60°C. on each of three successive days. Use the heated cyst fluid as antigen in test.

**B9\*744 ACUTE POLIOMYELITIS.**—(1) Make a 5 per cent suspension in sterile T. W. of brain and spinal cord of monkeys that have died of poliomyelitis. (2) Filter through a Berkefeld candle. (3) Add 2 per cent trypsin. (4) Leave 3 hr. at R. T. (5) Add 0.5 per cent phenol. (6) Dilute 1.5 for use as antigen in test.

**B9\*745 SCHISTOSOMIASIS.**—(1) Extract infected<sup>1</sup> snails' livers with abs. alc. 1 c.c. to each triturated liver. (2) Keep 24 hr. at 37°C. with occasional shaking. (3) Filter. (4) Evaporate the filtrate to a powder. (5) Make a suspension of the powder of strength 2.5 mgm. per c.c. of 0.5 per cent carbolized, 0.85 S.S.S. (6) Shake 30 min. on a mechanical shaker. (7) Filter. (8) Use the fresh filtrate in test.

**Notes.**—<sup>1</sup>The antigen may apparently be prepared from the livers of snails infested with schistosome cercariae, whether of *Sch. hematobium* or of *Sch. bovis*. This antigen has also been used for the detection of infection of sheep with liver flukes.

**B9\*75 BLOOD: B9\*751 STANDARDIZATION ANTI-SERA.**—(1) Set up a series of 10 small T.T. containing 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, c.c. highly potent<sup>1</sup> inactivated<sup>2</sup> 1-10 anti-serum with 0.1 c.c. 1-1000 fresh antigen serum, and 1 c.c. 1-20 fresh G.P. complementing serum. (2) Make up to vol. 2 c.c. with 0.85 S.S.S. (3) Set up four control T.T. containing 1, 0.8, 0.4, 0.2 c.c. inactivated<sup>2</sup> 1-10 anti-serum with 1 c.c. 1-20 fresh complementing G. P. serum. (4) Make up to vol. 2 c.c. with 0.85 S.S.S. (5) Set up a fifth control T.T. containing 0.1 c.c. 1-1000 fresh antigen serum and 1 c.c. 1-20 fresh complementing G.P. serum. (6) Make up to vol. 2 c.c. with 0.85 S.S.S. (7) Keep 1 hr. at 37°C. (8) Add 2 M.H.D. (**B9\*713**) inactivated anti-sheep serum and 1 c.c. 2.5 per cent washed sheep erythrocyte suspension. (9) Keep 90 min. at 37°C. (10) Examine. (11) Use in the test 2 minimum inhibiting<sup>3</sup> doses of anti-serum.<sup>4</sup> (12) Test all anti-sera in the same way.

**Notes.**—<sup>1</sup>Capable of fixing complement with 0.0001 c.c. of its antigen. <sup>2</sup>30 min. at 56°C. <sup>3</sup>The smallest quantity of anti-serum which effects complete fixation of complement and consequently inhibits hemolysis. <sup>4</sup>In forensic practice a human blood anti-serum is the first one to be tried.

**B9\*752 BLOOD STAIN TEST.**—(1) Prepare blood stain antigen (**B10\*821, A3\*418, A3\*419**). (2) Set up six small T.T. containing 0.1, 0.2, 0.4, 0.6, 0.8 and 1 c.c. blood stain antigen respectively. (3) Add 2 minimum inhibiting doses of anti-serum and 1 c.c. 1-20 fresh G.P. complementing serum. (4) Bring the total vol. up to 3 c.c. in each T.T.

with 0·85 S.S.S. (5) Set up three small control T.T., No. 1 containing 1 c.c. blood stain antigen 1 c.c. 1-20 fresh G.P. complementing serum, and 1 c.c. 0·85 S.S.S., No. 2 containing 2 minimum inhibiting doses of anti-serum, 1 c.c. 1-20 fresh G.P. complementing serum and sufficient 0·85 S.S.S. to bring the total vol. to 3 c.c., No. 3 containing 1 c.c. 1-20 fresh G.P. complementing serum and sufficient 0·85 S.S.S. to bring the total vol. to 3 c.c. (6) Keep all the T.T. 1 hr. at 37C. or, in preference to this, in the ice chest overnight at 8C. (7) Add 2 minimum hæmolytic doses of inactivated anti-sheep serum and 1 c.c. 2·5 per cent washed sheep erythrocyte suspension. (8) Keep 1 hr. at 37C. or until the control T.T. show hæmolysis. (9) Read the results.<sup>1</sup> (10) Keep the T.T. in the ice chest overnight and read the results<sup>1</sup> again.

**Notes.**—<sup>1</sup>Inhibition of hæmolysis (fixation of complement) with 0·1 c.c. up to 1 c.c. of blood stain antigen demonstrates that this test antigen is specific for the particular anti-serum used. In the case of human anti-serum a like result could only be given by the blood of the higher apes. A necessary control to the whole test is the simultaneous use of dried stains of blood of known species of animals and particularly of human blood.

**B9·76 MEAT: B 9·761.**—(1) Extract 30 grm. finely minced meat 12 hr. in the cold with 30 c.c. 0·85 S.S.S. (2) Filter through hard paper. (3) Use the filtrate<sup>1</sup> undiluted and in dilution 1-10 as antigen in the test. (4) Set up a series of five small T.T. :—Nos. 1 and 2 containing 0·5 c.c. undiluted and 1-10 diluted antigen, with 0·1 c.c. anti-serum<sup>2</sup> and 0·5 c.c. 1-10 fresh G.P. complementing serum, No. 3 containing 1 c.c. undiluted antigen with 0·5 c.c. 1-10 fresh G.P. complementing serum, No. 4 containing 0·2 c.c. anti-serum with 0·5 c.c. 1-10 fresh G.P. serum, and No. 5 containing 0·5 c.c. 1-10 fresh G.P. serum. (5) Bring the fluid in all the T.T. up to a vol. of 1·5 c.c. by the addition of 0·85 S.S.S. (6) Keep 1 hr. at 37C. (7) Add to each T.T. 2 M.H.D. (**B9·713**) of inactivated hæmolytic serum and 0·5 c.c. 5 per cent erythrocyte suspension. (8) Keep at 37C until hæmolysis is complete in the controls. (9) Read the results.

**Notes.**—<sup>1</sup>The clear filtrate represents approximately 1-100 dilution of protein.

<sup>2</sup>The anti-serum is obtained by inoculating rabbits with the serum, or a meat extract of the species of animal whose flesh is to be tested for.

**B9·762 SAUSAGE MEAT.**<sup>1</sup>—(1) Mince finely 30 grm. sausage meat. (2) Extract<sup>2</sup> 12 hr. in the cold with 30 c.c. 0·85 S.S.S. (3) Shake wel. (4) Centrifugalize. (5) Use the S.N.F. as antigen in test.

**NOTES.**—<sup>1</sup>The test which is usually required is for the presence of horse flesh. <sup>2</sup>If the sausage meat is very fat it should be extracted with ether first. Antiformin extracts also may be used.

**B9·8 CYTOLYTIC ACTION : B9·81 CARCINOMA : B9·811.—**

(1) Use non-degenerated portions of carcinomatous tissue from the edge of a growth.<sup>1</sup> (2) Cut up into small pieces and wrap in a cloth. (3) Squeeze out the fluid from the material into 0·6 per cent S.S.S. to which 1 per cent sod. fluoride has been added = cell suspension. (4) Wash by centrifugalizing 3 times with 0·6 per cent S.S.S. (5) Make a suspension of the deposit with 1 per cent sod. fluoride. (6) Set up a mixture :—test serum 10 : cell suspension 1 : 0·5 per cent sod. fluoride 1. (7) Set up controls. (8) Keep 24 hr. at 37°C. (9) Make a count<sup>2</sup> of the cells before and after incubation at 37°C.

**Notes.**—<sup>1</sup>Used in diagnosis of carcinoma. <sup>2</sup>The cells are disintegrated by normal sera but not by carcinomatous sera.

**B9·812.—**(1) Use non-degenerated portions of carcinomatous tissue.

(2) Grind up in a mortar to make a cell suspension with 5 vol. 1 per cent di-sod. phosphate. (3) Filter through several layers of gauze. (4) Allow the cells to settle. (5) Decant the S. N. F. (6) Suspend the cells in 0·6 per cent sod. chloride. (7) Allow the cells to settle. (8) Decant the S. N. F. (9) Suspend the cells in 1 per cent sod. fluoride.<sup>1</sup> (10) Set up a mixture :—freshly collected test serum 10 : cell suspension 1 : 0·5 per cent sod. fluoride 1. (11) Set up a mixture :—freshly collected normal serum 10 : cell suspension 1 : 0·5 per cent sod. fluoride 1. (12) Place samples of the mixtures on the platform of a blood counting chamber. (13) Lute the coverglass with vaselin to prevent evaporation. (14) Keep 24 hr. at 37°C. (15) Compare counts of the cells before and after incubation.

**Note.**—<sup>1</sup>Neutralize to alizarin until only a trace of the violet colour remains.

**B10 BLCOD SERUM REACTIONS : B10·1 DIGESTIVE ACTION.<sup>1</sup>**

**Notes** —<sup>1</sup>Syn. ferment action. Proteolytic action. Ninhydrin test. Polarimetric test.

**B10·11 DIALYZATION TEST.<sup>1</sup>**

**Notes.**—<sup>1</sup>Used to be applicable for the demonstration of special digestive ferment action in pregnancy, dementia præcox, exophthalmic goitre, malignant disease, epilepsy, tetra sodium infection, ascariis lumbricoides infection, diphtheria, anthrax, typhoid fever, trypanosomiasis, spirochaetosis, etc.

**B10·111 GLASSWARE PREPARATION.**—(1) Wash all glassware in water followed by alcohol and ether. (2) Wash finally with D.W. (3) Sterilize (§9·2) by hot air.

**B10·12 NINHYDRIN REAGENT.**—(1) Make, with sterile precautions, a 1 per cent sol. in D.W. (2) Preserve in a cool dark place.

**B10·113 SUBSTRATE.**—(1) Cut the substrate material up into small pieces and wash well in running water. (2) Free the tissue, by handling and squeezing, from blood and blood clot. (3) Grind up the material in a mortar. (4) Remove connective tissue. (5) Wash until the material is quite free of blood. (6) Make a 1 per cent suspension in D.W. (7) Add 5 drops glacial ascorbic acid per litre. (8) Boil 10 min. (9) Wash the coagulated material with D.W. until free of acid. (10) Resuspend in D.W. and boil 10 min. (11) Continue the process six times in succession. (12) Suspend in only a small quantity<sup>1</sup> of water. (13) Boil 5 min. (14) Filter off the water for test. (15) Add to 5 c.c. filtrate in a T.T. 1 c.c. ninhydrin sol. (16) Boil 1 min. (17) Note the absence of any colouration<sup>2</sup> after 30 min. (18) Preserve the solid material in a sterile jar containing D.W. and covered with toluol.<sup>3</sup>

**Notes.**—<sup>1</sup>Just sufficient water to prevent danger of burning with boiling, and to give sufficient fluid for test. <sup>2</sup>The washing and boiling must be repeated if any trace of colour appears. <sup>3</sup>Only sterile forceps should be used to remove tissue from the jar and once removed none should ever be put back. The process of preparation lasts several hrs. and if interrupted the material should be covered with toluol.

**B10·114 DIALYZING SHELLS PREPARATION.**—(1) Soak new shells in sterile D.W. until they are pliable. (2) Boil for 30 sec. (3) Place in a jar of sterile D.W. containing a few drops of chloroform and covered with toluol. (4) Remove for use with clean sterile forceps only.

**B10·115 DIALYZING SHELLS, IMPERMEABILITY TO ALBUMIN.**—(1) Place fresh blood serum<sup>1</sup> free from all traces of hæmoglobin in each shell, taking care not to soil the outside of the shell. (2) Place the shell and its contents in a sterile glass cylinder plugged with cotton wool and containing 20 c.c. sterile D.W. (3) Cover the fluid in the shell with toluol and also the water surrounding the shell. (4) Keep 20 hr. at 37°C. (5) Pipette off, with sterile precautions, 10 c.c. dialysate from beneath the toluol layer into a clean sterile T.T. (6) Add to the T.T. 2·5 c.c. 33 per cent sod. hydroxide. (7) Mix carefully. (8) Add 1 c.c. 0·2 per cent copper sulphate to form a layer on top of the fluid in the T.T. (9) Note<sup>2</sup> the formation of any colouration at the interface between the two fluids. (10) Reject any shells which are permeable.

**Notes.**—<sup>1</sup>A 5 per cent sol. of white of egg may be used. <sup>2</sup>No colouration should appear. The occurrence of a violet tint shows permeability of the shell to albumin.

**B10-116 DIALYZING SHELLS PERMEABILITY TO PEPTONE.**—(1) Place 1-1000 peptone sol. in each shell, taking great care not to soil the outside of the shell. (2) Place the shell and its contents in a sterile glass cylinder plugged with cotton wool and containing 20 c.c. sterile D.W. (3) Cover the fluid in the shell with toluol and also the water surrounding the shell. (4) Keep 24 hr. at 37°C. (5) Pipette off, with sterile precautions, 10 c.c. dialysate from beneath the toluol layer into a clean sterile T.T. (6) Add 0.2 c.c. ninhydrin sol. (7) Boil for exactly one min. (8) Leave 30 min. (9) Note the colour<sup>1</sup> of the fluid in the T.T. (10) Reject any shells which are not permeable.

**Notes.**—<sup>1</sup>It should show a deep blue; if it does not, then the shell is impermeable, or only partly permeable to peptone.

**B10-117 TEST.**—(1) Use a perfectly clear test serum, free of erythrocytes, within 12 hr. of obtaining the blood. (2) Remove a sufficient amount of the substrate material with sterile forceps. (3) Wash in sterile D.W. to remove toluol. (4) Boil 2 min. with 5 vol. sterile D.W. and test the water with ninhydrin. (5) Place the substrate material on sterile filter paper. (6) Squeeze to remove excess water. (7) Weigh. (8) Set up three shells for the test. (9) Place 0.5 grm. substrate material in Nos. 1 and 2 shells. (10) Add 1.5 c.c. test serum to No. 1 and No. 3 shells. (11) Add 1.5 c.c. sterile D.W. to No. 2 shell. (12) Hold each shell shut with sterile forceps and wash the outside well with sterile D.W. (13) Place each shell<sup>1</sup> and its contents in a sterile glass cylinder plugged with cotton wool and containing 20 c.c. sterile D.W. (14) Cover the fluid in the shell with toluol and also the water surrounding the shell. (15) Keep 24 hr. at 37°C. (16) Set up controls with normal serum and with a known positive serum. (17) Pipette off with sterile precautions 10 c.c. dialysate from beneath the toluol layer into a clean sterile T.T. (18) Boil with 0.2 c.c. ninhydrin sol. exactly one min. (19) Leave 30 min. (20) Note the colour of the fluid in the T.T.<sup>2</sup>

**Notes.**—<sup>1</sup>The shell should project at least 1-4th in. above the level of the surrounding fluid. <sup>2</sup>The dialysate of No. 2 and No. 3 shells should be without colour or show only the faintest blue tinge. The dialysate of No. 1 shell shows a deep violet blue colouration when the reaction is strongly positive, or a fainter blue when weakly positive. The reaction is negative if this dialysate is without colour, or shows only the faintest blue tinge. A pinkish or brownish yellow discolouration is not a positive reaction.

#### **B10-112 POLARIZATION TEST.<sup>1</sup>**

**Notes.**—<sup>1</sup>V. B10-1101 for applicability.

**B10-121.**—(1) Place in the polarization tube. 10 per cent standard peptone<sup>1</sup> 1 c.c.; test serum<sup>2</sup> 1 c.c.; 0.85 S.S.S. to fill the polarization tube.

(2) Keep 1 hr. at 37C. (3) Set up controls with known normal and known positive sera. (4) Read the degree of rotation<sup>3</sup> hourly for 6 hr. and then after 36 to 48 hr.

**Notes.**—<sup>1</sup>A specially prepared peptone. <sup>2</sup>Must be absolutely free of hæmoglobin and erythrocytes. <sup>3</sup>The rotation should show little change during the first two hr. Cleavage is usually evident in positive sera at the end of 6 hr. and may amount to 0.05 to 0.02 degrees. It seldom amounts to more than 0.03 degrees in normal sera.

**B10.2 EPIPHANIN REACTION: B10.21 SYPHILIS.**—(1) Use syphilitic liver antigen (**A3.611**), fresh unheated serum, N-1 sulphuric acid, N-1 barium hydroxide,<sup>1</sup> and 1 per cent alc. phenolphthalein sol. containing 10 per cent strontium chloride. (2) Set up 5 T.T. containing, Nos. 1 to 4, 1 c.c. appropriate antigen dilution,<sup>2</sup> No. 5, 1 c.c. 0.85 S.S.S. (3) Add to Nos. 1 to 3, 0.1 c.c. of a series of dilutions of test serum and to No. 5, 0.1 c.c. 0.85 S.S.S. (4) Add to each of the T.T. of the entire series very carefully 2 c.c. N-1 sulphuric acid, 2 c.c. barium hydroxide, and 0.1 c.c. phenolphthalein strontium chloride mixture. (5) Add to No. 4 T.T.<sup>3</sup> in which the reaction will already have taken place, 0.1 c.c. of the same dilution of test serum as was added to T.T. No. 2. (6) Read the results.<sup>4</sup>

**Notes.**—<sup>1</sup> Prepared from a saturated sol. The sol. must show no trace of cloudiness. *E.g.*, 1-10,000. <sup>2</sup>The control T.T. with which comparisons of colour are made. <sup>3</sup>A positive result is obtained if T.T. Nos. 1 to 3 show lessened alkalinity than No. 4, *i.e.*, a lighter pink colour. A negative result is obtained if T.T. Nos. 1 to 3 are more alkaline than No. 4, *i.e.*, show a more intense pink than No. 4 T.T. The exact degree of increase of H-ion concentration produced, when the test serum is specific for the antigen may be determined by titration.

### **B10.3 FLOCCULATION REACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup> A reaction used for the detection of syphilitic sera.

**B10.31: B10.311 ANTIGEN.**—(1) Digest together for 1 hr. :—serum<sup>1</sup> 1; 5 per cent trypsin 1. (2) Prepare :—serum digest 1; methyl alc. 50. (3) Keep 15 min. at 37C. (4) Shake gently to mix. (5) Centrifugalize. (6) Pipette off the clear S.N.F. for use.

**Notes.**—<sup>1</sup> Test sera, and known positive and normal sera.

**B10.312 TEST.**—(1) Set up a number of racks each containing 8 small T.T., one rack for each serum. (2) Add to each T.T. of each rack 8 vol. of serum. (3) Add to each T.T. 1 vol. 5 per cent platinum chloride. (4) Compare the test sera T.T. with the known positive and known normal sera T.T. for rate of appearance<sup>1</sup> of flocculation.

**Notes.**—<sup>1</sup> In the case of the normal serum a distinct fine precipitate appears almost immediately, whereas in the case of a syphilitic serum the fluid remains clear for an

appreciable time after the reagent has been added. The test is dependent on the assumed deficiency of syphilitic serum in lecithin and the precipitation of the latter by platinum chloride.

**B10·32 : B10·321.**—(1) Prepare :—fresh unheated test serum 5 c.c.; D.W. 2 c.c.; 25 per cent chemically pure nitric acid of S.G. 1·149, 3 c.c. (2) Shake to mix. (3) Keep 10 min. at R.T. (4) Add 16 c.c. D.W. (5) Invert to mix. (6) Keep 10 min. at R.T. (7) Invert to mix. (8) Observe precipitate.<sup>1</sup>

**Notes.**—<sup>1</sup> A precipitate persists in the case of a syphilitic serum but goes into sol. in the case of a normal serum. Slight opalescence is taken as positive.

**B10·322.**—(1) Prepare No. 1 sol.<sup>1</sup>:—96 per cent alc. 1; D.W. 2. (2) Prepare No. 2 sol.<sup>1</sup>:—96 per cent alc. 2; D.W. 5·5. (3) Add 0·2 c.c. inactivated test serum to 3 c.c. of each of No. 1 and No. 2 sol. (4) Observe flocculation<sup>2</sup> at the end of 10 and 60 min.

**Notes.**—<sup>1</sup> Must be absolutely neutral. <sup>2</sup>Positively syphilitic sera show flocculation with both sol.

**B10·323.**—(1) Determine the highest dilution of 0·1 per cent lactic acid which when added in the quantity 2 c.c. to 0·2 c.c. inactivated test serum still gives a definite flocculation. (2) Compare with a known normal serum.

**B10·33 : B10·331.**—(1) Prepare :—inactivated test serum<sup>1</sup> 0·4; fresh 2 per cent sod. glycocholate<sup>2</sup> 0·2; 1·19 glycholate cholesterin sol.<sup>3</sup> 0·2. (2) Shake gently to mix. (3) Keep 20 hr. at R.T. (4) Examine for flocculation.

**Notes.**—<sup>1</sup> Hemoglobin-free, inactivated 30 min. at 55°C. <sup>2</sup>In distilled water. <sup>3</sup>Make a stock sol. sod. glycocholate 2; cholesterin 0·4; 95 per cent alc. 100. Dilute 1·19 with D.W.

**B10·34 : B10·341 CYTOZYME.**<sup>1</sup>—(1) Use alc. extract of G.P.'s heart.

**Notes.**—<sup>1</sup> Syn. thrombozyme, thrombokinase.

**B10·342 SEROZYME.**—(1) Prepare this serum which is the serum exuding from oxalate plasma coagulated with calc. chloride, 2 or 3 hr. before use. (2) Dilute 1·5, 1 hr. before use.

**B10·343 CALC. CHLORIDE.**—(1) Prepare :—1 per cent calc. chloride 1; 0·85 S.S.S. 20.

**B10·344 OXALATE PLASMA DILUTION.**—(1) Prepare :—oxalate plasma 1; 1 per cent sod. oxalate 1; 0·85 S.S.S. 3.

**B10·345 TEST.**—(1) Set up 4 T.T. containing 0·1 c.c. inactivated serum. (2) Add to T.T. Nos. 1 to 3, 0·1 c.c. 1·40, 80, 160 cytozyme

(3) Add to No. 4 T.T., 0.1 c.c. 0.85 S.S.S. (1) Keep 30 min. at R.T. (5) Add to each T.T. 1 c.c. calc. chloride sol. and 0.5 c.c. serozyme. (6) Shake to mix. (7) Keep 15 min. at R.T. (8) Add 1 c.c. oxalate plasma dilution. (9) Incline the T.T. at intervals to determine<sup>1</sup> the time of coagulation. (10) Set up control T.T. in which the serum is replaced by 0.85 S.S.S. to determine<sup>1</sup> the coagulation in these T.T.

**Notes.**—<sup>1</sup> Coagulation ought to appear in the salt control T.T. in 2 to 4 min.; later, in the case of normal sera T.T., in 10 to 60 min. or not at all, with syphilitic sera.

**B10.35 : B10.351.** (1) Prepare :—fresh clear test serum 1 ; D.W. 3. (2) Keep 15 hr. at R.T. (3) Set up controls with normal and known positive sera. (4) Observe flocculation.<sup>1</sup>

**Notes.**—<sup>1</sup> Flocculation occurs only in the case of syphilitic sera.

**B10.36 : B10.361.** (1) Prepare :—inactivated serum 0.2 c.c.; organ extract<sup>1</sup> diluted<sup>2</sup> with D.W., 1.5 c.c. (2) Shake to mix. (3) Keep 60 min. at 37C. (4) Add 25 c.c. D. W. at 37C. (5) Shake to mix. (6) Keep 16 hr. at 37C. (7) Observe flocculation.<sup>3</sup>

**Notes.**—<sup>1</sup> Human heart. <sup>2</sup>The dilution varies with the extract. <sup>3</sup>Normal sera precipitate, whilst positive sera show only opalescence.

**B10.362.**—(1) Make a 1.8 dilution of alc. extract of human heart by very slow addition of D.W. (2) Set up a series of small T.T. each containing 0.2 c.c. of inactivated known normal serum, and 1 c.c. antigen dilution. (3) Leave 24 hr. (4) Add 1 c.c. sol. of sod. chloride varying from 1.4 to 2.4 per cent to the T.T. (5) Note the highest dilution of sod. chloride which dissolves the precipitate which is formed on mixture of serum and antigen. (6) Set up two small T.T. each containing 0.2 c.c. inactivated test serum. (7) Add to No. 1 T.T. 0.8 c.c. and to No. 2 T.T. 1 c.c. antigen dilution. (8) Keep 20 to 24 hr. at 37C. (9) Shake to distribute the precipitate. (10) Add to both T.T. the selected dilution of sod. chloride. (11) Observe flocculation.<sup>1</sup>

**Notes.**—<sup>1</sup> Persists in the case of syphilitic sera.

**B10.37 NOGUCHI : B10.371.**—(1) Add 1 c.c. 10 per cent butyric acid in 0.85 S.S.S. to 0.2 c.c. clear unstained cerebro-spinal fluid. (2) Heat gently to boil. (3) Add quickly 0.2 c.c. N-1 sod. hydroxide. (4) Boil for a few sec. (5) Keep 1 hr. at R.T. (6) Examine for flocculation.<sup>1</sup>

**Notes.**—<sup>1</sup> A positive reaction is indicative of a true meningitis, e.g., tubercular.

**B10.38 : B10.381.**—(1) Add 1 c.c. 1.5 inactivated test serum to 0.2 c.c. 1 per cent lecithin in 0.5 per cent carbolized 0.85 S.S.S. (2) Keep 2 hr. at 37C. (3) Keep 24 hr. at R.T. (4) Observe flocculation.<sup>1</sup>

**Notes.**—<sup>1</sup> Occurs only in a syphilitic serum.

**B10-382.**—(1) Prepare :—inactivated clear serum<sup>1</sup> 1 : 1 per cent pure sol. glycocholate in D.W. 1. (2) Keep at R.T. 20 hr. (3) Examine for coarse flocculation.

**Notes.**—<sup>1</sup> Use test, normal, and known positive sera.

**B10-39 SACHS AND GEORGI : B10-391 ANTIGEN.**—(1) Extract 1 gm. ox heart with 5 c.c. 96 per cent alc. (2) Add to 100 c.c. of this extract, 200 c.c. 96 per cent alc. and 13 c.c. 1 per cent cholesterol. (3) Dilute this mixture at the time of use with an equal quantity of 0.85 S.S.S. and mix. (4) Add rapidly 0.85 S.S.S. to an amount equal to four times the vol. of the mixture. (5) Use as antigen dilution in the test.

**B10-392 TEST.**—(1) Prepare :—antigen dilution 1 : 1.10 inactivated test serum 2. (2) Keep 2 hr. at 37°C. (3) Keep 18 to 20 hr. at R.T. (4) Observe flocculation.<sup>1</sup> (5) Set up controls.

**Notes.**—<sup>1</sup>Positively syphilitic sera show numerous small particles which deposit and leave a clear S.S.F. Negative sera are clear or faintly opalescent.

#### **B10-4 HÆMOLYTIC ACTION : B10-41 HÆMOLYTIC SERUM STANDARDIZATION.<sup>1</sup>**

**Notes.**—<sup>1</sup>V. also B9-713.

**B10-411.**—(1) Mix increasing quantities but in equal vol. of inactivated<sup>1</sup> hæmolytic serum with 1 c.c. 5 per cent suspension of suitable erythrocytes and 0.1 c.c. fresh G.P. serum.<sup>2</sup> (2) Keep 2 hr. at 37°C. (3) Read the result.<sup>3</sup>

**Notes.**—<sup>1</sup>30 min. at 56°C. <sup>2</sup>The quantity 0.1 c.c. G.P. serum has, by experience, been found to be the most suitable for 1 c.c. 5 per cent suspension of washed erythrocytes. <sup>3</sup>The smallest quantity of hæmolytic serum, which still gives complete hæmolysis, is the unit generally adopted.

**B10-412.**—(1) Set up a series of small T.T. containing, say, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 c.c. 1-100 inactivated<sup>1</sup> hæmolytic serum for sheep erythrocytes together with 1 c.c. 1-20 fresh G.P. complementing serum, and 1 c.c. 2.5 per cent washed erythrocyte suspension. (2) Set up 3 control small T.T. containing :—No. 1, 1 c.c. 1-100 inactivated<sup>1</sup> sheep hæmolytic test serum and 1 c.c. 2.5 per cent washed sheep erythrocyte suspension, No. 2, 1 c.c. 1-20 fresh G.P. complementing serum and 1 c.c. 2.5 per cent sheep erythrocyte suspension, No. 3, 1 c.c. sheep erythrocyte suspension. (3) Make up the vol. in all the T.T. to 3 c.c. with 0.85 S.S.S. (4) Keep 1 hr. at 37°C. (5) Note the T.T. in the test series which shows complete hæmolysis with the smallest content of hæmolytic serum. (6) Record this amount of hæmolytic serum as the minimum hæmolytic dose.<sup>2</sup>

**Notes.**—<sup>1</sup>30 min. at 56°C. <sup>2</sup>The unit.

**B10·42 TRANSFUSION TESTS.<sup>1</sup>**

**Notes.**—<sup>1</sup>See also **B8·33**.

**B10·421.**—(1) Prepare 10 per cent suspension washed erythrocytes (**A3·411**, **A3·412**) in 0·85 S.S.S. from donor and recipient. (2) Prepare :—erythrocyte suspension donor 1 ; serum recipient 4. (3) Prepare :—erythrocyte suspension recipient 1 ; serum donor 4. (4) Prepare :—erythrocyte suspension donor 1 ; serum donor 4. (5) Prepare :—erythrocyte suspension 1 ; serum recipient 4. (6) Add 1 c.c. 0·85 S.S.S. to each of the mixtures. (7) Keep 2 hr. at 37C. (8) Note the result as regards hæmolysis and agglutination. (9) Keep overnight in the ice chest. (10) Read the results again.

**B10·422.**—(1) Set up a series of six small T.T. containing :—No. 1, 4 vol. fresh donor's serum and 1 vol. recipient's erythrocyte suspension ; No. 2, 4 vol. fresh recipient's serum and 1 vol. donor's erythrocyte suspension ; No. 3, 4 vol. fresh donor's serum and 1 vol. donor's erythrocytes ; No. 4, 4 vol. fresh recipient's serum and 1 vol. recipient's erythrocyte suspension ; No. 5, 4 vol. 0·85 S.S.S. and 1 vol. donor's erythrocyte suspension ; No. 6, 4 vol. 0·85 S.S.S. and 1 vol. recipient's erythrocyte suspension. (2) Add 20 vol. 0·85 S.S.S. to each T.T. (3) Shake gently to mix. (4) Keep 2 hr. at 37C. and inspect the T.T. every 30 min. for the occurrence of hæmolysis or agglutination.

**Notes.**—<sup>1</sup>Test for isohæmolysin and isoagglutinin (**B8·331**). The particular grouping to which one's own blood belongs should be known (**B8·332**).

**B10·5 MEIOSTAGMIN REACTION.<sup>1</sup>**

**Notes.**—<sup>1</sup>Dependent on the fact that a lowering of surface tension results from the interaction of antigen and antibody. The greater the surface tension the larger the drops formed and therefore the less their number.

**B10·51 ANTIGEN : B10·511 BACTERIAL.**—(1) Wash off the 48-hr. growth of several agar cultures with 5 c.c. 0·85 S.S.S. per culture. (2) Cover the suspension obtained with toluol. (3) Shake for several hr. (4) Keep 48 hr. at 37C. (5) Filter through a sterile Berkefeld or Chamberland F candle. (6) Use the filtrate as antigen.

**B10·512 BACTERIAL.**—(1) Dry the 48-hr. growth on agar *in vacuo* over sulphuric acid or calc. chloride. (2) Grind up 0·5 gm. of the powder obtained with 20 c.c. methyl alc. (3) Filter through a sterile Berkefeld or Chamberland F candle. (4) Use the filtrate as antigen.

**B10·513 CANCER.**—(1) Mince finely undegenerated portions of tumour. (2) Dry at 37C. or *in vacuo* over sulphuric acid or calc. chloride. (3) Grind up the powder. (4) Extract, with occasional shaking, for

24 hr. at 50°C. with methyl alc. using 5 c.c. per gram. (5) Filter while hot. (6) Allow to cool. (7) Filter through Schleicher and Schull No. 590 paper. (8) Preserve this antigen carefully from all access of moisture.<sup>1</sup>

**Notes.** —<sup>1</sup>All pipettes, etc., used must be thoroughly dry.

**B10·514 CANCER.**—(1) Extract 0·5 gram. lecithin with 50 c.c. acetone 24 hr.<sup>1</sup> at 50°C. (2) Filter and refilter till clear through filter paper. (3) Dilute<sup>2</sup> for use to an extent that will just not cause a marked reduction of drop number in normal serum.

**Notes.** —<sup>1</sup>A 'synthetic' cancer antigen. <sup>2</sup>1:50 to 1:100.

**B10·515 SYPHILITIC.**—(1) Extract 0·5 gram. dried and powdered syphilitic liver with 50 c.c. abs. alc. for 24 hr. at 37°C., with frequent shaking. (2) Filter. (3) Concentrate filtrate to 10 c.c.

**B10·52 ANTIGEN STANDARDIZATION.**—(1) Set up a series of T.T. each containing 1 vol. of the dilutions of antigen 1:5, 10, 20, . . . . . 200. (2) Add 9 vol. fresh normal serum diluted 1:20 with 0·85 S.S.S. to each T.T. (3) Set up a T.T. containing 1 vol. of D.W. and 9 vol. fresh 1:20 normal serum. (4) Shake to mix. (5) Keep 2 hr. at 37°C. (6) Determine the drop number of 1 c.c. of each of the mixtures. (7) Use in the test the lowest dilution of antigen which does not increase the drop number for normal serum beyond 1 drop in a total of 1 c.c. over that given by the mixture in which D.W. replaces antigen dilution.

**B10·53.**—(1) Set up the stalagmometer rigidly fixed in a vertical position. (2) Use scrupulously clean glassware and do not plug with cotton wool. (3) Determine the drop number of 1 c.c. of fresh test serum diluted with 0·85 S.S.S. (4) Set up three clean T.T. each containing 1 vol. of the selected antigen dilution. (5) Add to No. 1 T.T. 9 vol. 1:20 test serum, to No. 2 T.T. 9 vol. 1:20 normal serum, to No. 3 T.T. 9 vol. known positive serum. (6) Set up a control T.T. containing 1 vol. D.W. and 9 vol. 1:20 test serum. (7) Keep all the T.T. 2 hr. at 37°C. (8) Allow to cool. (9) Determine the drop number of 1 c.c. of each mixture. (10) Record the result.<sup>1</sup> (11) Clean out the stalagmometer with D.W., alc., and ether for each successive measurement, getting rid of the ether by means of a current of hot air.

**Notes.** —<sup>1</sup>Increase of the drop number by more than 2 over that of the control mixture when D.W. takes the place of antigen dilution may be regarded as a positive result. This increase will seldom exceed 8.

**B10·6 OPSONIC ACTION: B10·61 ANTIGEN: B10·611.** (1) Make a suspension of a young<sup>1</sup> agar culture<sup>2</sup> in 0·85 S.S.S. (2) Allow

unresolved masses to settle. (3) Aspirate the upper layer of suspension into the bulb of a capillary pipette. (4) Seal through the capillary stem just below the bulb, and so obtain a small T.T. containing the suspension. (5) Centrifugalize, if necessary, to remove clumps.

**Notes.**—<sup>1</sup>18 hr. or less. Gonococcus cultures and cultures of coliform organisms should not be more than 12 hr. old. <sup>2</sup>In the case of dangerous organisms kill before using in test by means of formalin.

**B10·612 STAPHYLOCOCCUS.**—(1) Transfer a large loopful of a young agar culture to an empty watch-glass. (2) Grind up in the watch-glass with additions of 0·85 S.S.S. drop by drop as required. (3) Centrifugalize the suspension to remove unresolved clumps.

**B10·613 PNEUMOCOCCUS.**—(1) Use D.W. instead of 0·85 S.S.S. in the case of freshly isolated strains of pneumococcus, which are not easily phagocytosable.

**B10·614 B. TUBERCULOSIS.**—(1) Sterilize a 10*l.* glycerin broth culture by short heating in the steamer. (2) Collect the bacterial growth on filter paper. (3) Wash with 0·85 S.S.S. (4) Keep the filter paper and growth at 37C. to dry. (5) Transfer the dry growth to a stoppered bottle and store. (6) Place about 10 mgm. dry growth in an agate mortar. (7) Grind up the growth, with the addition of 1 per cent S.S.S., as necessary, to make a thick suspension. (8) Centrifugalize the suspension for 2 min. to remove unresolved clumps. (9) Transfer the S.N.F. to a clean centrifuge tube. (10) Centrifugalize<sup>1</sup> again for such time as is necessary to produce a suitable suspension. (11) Test the suitability of the suspension by mixing 1 unit vol. suspension with 1 unit vol. normal serum and 1 unit vol. of washed leucocytes, incubating 15 min., and making films.<sup>2</sup>

**Notes.**—<sup>1</sup>In the absence of a centrifuge the clumps may be removed by filtration through filter paper. <sup>2</sup>The leucocytes should on an average contain about 2 bacilli.

**B10·62 LEUCOCYTE SUSPENSION: B10·621.**—(1) Fill a small centrifuge tube  $\frac{2}{3}$ rd full with 1·5 per cent freshly made sod. citrate. (2) Prick the constricted finger and receive the blood into the citrate sol. (3) Close the open end of the centrifuge tube with the thumb and invert several times to mix. (4) Centrifugalize carefully<sup>1</sup> to give a clear S.N.F. (5) Remove the S.N.F. (6) Fill up the tube with 1 per cent S.S.S. and invert several times to mix. (7) Centrifugalize carefully. (8) Repeat the process of removing S.N.F. and adding fresh 1 per cent S.S.S. (9) Remove the S.N.F. (10) Mix up erythrocytes and leucocytes of the sediment and use as leucocyte suspension.

**Notes.**—<sup>1</sup>Without excessive speed so as to avoid excessive compaction of leucocytes.

**B10'63 BLOOD SERUM : B10'631.**—(1) Take blood from the patient and from one or more normal individuals in glass capsules. (2) Allow the blood to coagulate. (3) Use the separated serum.<sup>1</sup>

**Notes.**—<sup>1</sup>The sera, if kept in the dark, will retain their potency for two or more days.

**B10'64 TEST : B10'641.**—(1) Draw up into a capillary pipette furnished with a rubber teat 1 unit vol. washed leucocytes, 1 vol. antigen suspension and 1 vol. serum<sup>1</sup> separated from each other by an air seal. (2) Blow out on to a clean slide and mix thoroughly. (3) Draw up the mixture into the pipette. (4) Seal the lower end of the pipette and remove the rubber teat. (5) Keep 15 min.<sup>2</sup> at 37°C. (6) Have in readiness a pile of roughened<sup>3</sup> slides. (7) Take a pair of these slides from the pile and wipe their upper surfaces to remove any adhering traces of emery dust. (8) Break off the tip of the capillary stem of a pipette containing the serum antigen leucocyte mixture at the appropriate time. (9) Blow out the contents of the pipette on to the extreme left hand corner of one of two slides. (10) Fit a rubber teat to the pipette. (11) Use it to mix the serum antigen leucocyte mixture. (12) Set out one drop of the mixture on the mixing slide itself, and another on the second slide. (13) Make films of these drops with a special glass spreader.<sup>4</sup> (14) Continue the procedure at the appointed times with each of the serum antigen leucocyte mixtures. (15) Pour a little formalin<sup>5</sup> into a watch-glass or salt cellar. (16) Place a slide with film, film side downwards, over the formalin for 3 sec. (17) Stain 30 sec. with undiluted carbol thionin.<sup>6</sup> (18) Wash and dry.<sup>7</sup> (19) Count the bacteria ingested in 100 leucocytes. (20) Calculate the opsonic index.<sup>8</sup>

**Notes.**—<sup>1</sup> Both test serum and a normal serum for the necessary comparison. <sup>2</sup>This time may be prolonged when the bacterial suspension is too thin, and gives too small a phagocytic inclusion, or it may be shortened when the preliminary trial test has shown that it gives too large a count or results in too rapid digestion of the bacteria with consequent unsatisfactory staining. <sup>3</sup>Lay a slide down on the bench and twirl it. Select that side, the convex, on which it spins most easily, for preparation with emery paper. Roughen the surface with fine Hubert's 00 emery paper. <sup>4</sup>To make a spreader:—make a nick with the glass-cutting knife about half way along the side of the slide. Grasp the two ends of the slide between the fingers and thumb of either hand and advancing the thumb of the right hand as far as, or a little beyond, the intended line of fracture to serve as a fulcrum, break the slide across by putting a transverse strain on it and at the same time exerting a pull in the longitudinal direction. In this way an ideally smooth edge for spreading films will be obtained. It should preferably be slightly concave. The edge of the spreader should be wiped on a dry cloth after spreading each pair and from time to time it may be dipped into a watch glass of water and carefully dried. <sup>5</sup>Or fix in sat. mercury bichloride for 1 min. and wash thoroughly

in water. <sup>6</sup>Thionin 0.25 : 1 per cent carbolic acid 100. <sup>7</sup>If even a trace of water is left on the film a smudge is developed in which the leucocytes are found partially decolourized. <sup>8</sup>The opsonic index is obtained by dividing the phagocytic count of the patient's blood by the phagocytic count of the control blood. When used for diagnostic purposes evidence should be sought of negative and positive phases occurring in sequence, *i.e.*, for fluctuation of values with a given organism, and not with others that are tested. Failing evidence of spontaneous inoculations giving rise to fluctuation, use one or other of the methods which are available for eliciting artificial auto-inoculations, *e.g.* hot bath, respiratory exercises, massage, or Bier's bandage, and take specimens of blood immediately before, half to one hour later, and again 6 and 24 hr. after the procedure which is designed to elicit such an auto-inoculation. Another index than that here described is the extinction or zero index in which the test serum is diluted to the point at which the phagocytosis induced is the same as that given by using 0.85 S.S.S. in place of the serum.

**B10.642 B. TUBERCULOSIS.** (1) Bring carbol fuchsin nearly to the boil in a large T.T. (2) Pour over the opsonic film. (3) Leave 10 min. (4) Wash and drain. (5) Pour on to the film 2.5 per cent. sulphuric acid and leave 20 sec. (6) Wash. (7) Pour on to the film 4 per cent ascitic acid. (8) Wash. (9) Pour on to the film alkaline methylene blue<sup>1</sup> and leave 30 sec. (10) Wash. (11) Shake to get rid of as much water as possible. (12) Dry between sheets of clean blotting paper. (13) Place on the top of an incubator or other warm place to dry.

**Notes.**—<sup>1</sup>Methylene blue 1; sod. carbonate 1; D.W. 200.

**B10.643 BACTERIA EASILY DISINTEGRATED.**—(1) Use the procedure for *B. tuberculosis*.

**B10.7 PRECIPITIN ACTION: B10.71 BACTERIA: B10.711 ANTIGEN.**—(1) Filter a 3-week bouillon culture through a new<sup>1</sup> Berkefeld candle. (2) Use the filtrate as antigen.

**Notes.**—<sup>1</sup>An old filter candle is apt to keep back antigen.

**B10.712 ANTIGEN.**—(1) Make a suspension of a 48-hr. agar culture with 10 c.c. 0.85 S.S.S. (2) Leave overnight at 37C. (3) Filter through a new Berkefeld candle. (4) Use the filtrate as antigen.

**B10.713 ANTIGEN.** (1) Make a suspension of a 48-hr. agar culture with 10 c.c. 0.85 S.S.S. (2) Add 3 c.c. N-4 hydrochloric acid or N-4 sod. hydroxide. (3) Boil 20 min. (4) Neutralize. (5) Filter through filter paper. (6) Use the clear filtrate as antigen. (7) Preserve after addition of 0.5 per cent phenol in a cool dark place.

**B10.714 ANTIGEN.**—(1) Filter through a new Berkefeld candle an 8-week bouillon culture. (2) Add 6 vol. 95 per cent alc. to the filtrate. (3) Filter the mixture through paper. (4) Press the precipitate retained on the paper between folds of filter paper. (5) Dry it at R.T.

(6) Dissolve in water. (7) Precipitate by adding solid ammon. sulphate to saturation. (8) Filter off the precipitate. (9) Re-dissolve the precipitate in water. (10) Salt out again by the addition of ammon. sulphate to saturation. (11) Wash the precipitate with sat. ammon. sulphate sol. (12) Press between folds of filter paper. (13) Re-dissolve in water. (14) Remove the excess of ammon sulphate from this sol. by repeated fractional addition of 95 per cent alc. (15) Precipitate the antigen finally with a large excess of alc.

**B10\*715 TEST.**—(1) Set up a series of 4 T.T. each containing 2 c.c. antigen **(B10\*711)**. (2) Add 0.05, 0.1, 0.5 and 1 c.c. test serum to each T.T. (3) Set up controls with known positive and negative precipitin sera. (4) Leave 6 hr.<sup>1</sup> at R.T. without shaking. (5) Note in which precipitation has taken place and its amount.

**Notes.**—<sup>1</sup> When the serum is strongly precipitating the clouding of the clear fluid should take place in 15 min.

**B10\*716 TEST.**<sup>1</sup>—(1) Use antigen **B10\*712** in place of **B10\*711**.

**Notes.**—<sup>1</sup> Tubes, instruments and serum must be sterile and all precautions for sterility taken owing to the fact that the reaction is much slower than with serum precipitin, and may take as much as 24 hr. to develop with the consequent possibility of bacterial growth and turbidity due to that cause.

**B10\*72 D. PNEUMONIÆ : B10\*721 ANTIGEN.**—(1) Place 6 c.c. pneumonic sputum in a T.T. (2) Keep at 100°C. till a more or less firm coagulum results. (3) Break up the coagulum with a glass rod. (4) Add 1 to 1.5 c.c. of 0.85 S.S.S. (5) Keep a few min. at 100°C. with frequent shaking. (6) Centrifugalize. (7) Use the clear S.N.F. in the test.

**B10\*722 ANTIGEN.**—(1) Dissolve pneumococcus sputum by the addition to it of 5 per cent antiformin.<sup>1</sup> (2) Boil. (3) Over neutralize while hot, with acid, to precipitate albumin. (4) Centrifugalize. (5) Pipette off the S.N.F. and neutralize. (6) Add several vol. alc. to produce a precipitate. (7) Centrifugalize. (8) Remove the alc. (9) Extract the sediment with about 1 c.c. 0.85 S.S.S. at 100°C. (10) Centrifugalize. (11) Use the clear S.N.F. as antigen for typing.

**Notes.**—<sup>1</sup> Bile may be used to dissolve pneumococci.

**B10\*723 ANTIGEN.**—(1) Wash test sputum with sterile 0.85 S.S.S. (2) Inject 1 c.c. intraperitoneally in a mouse. (3) Kill the mouse after 6 hr. (4) Wash out the peritoneum with 8 c.c. 0.85 S.S.S. (5) Centrifuge the turbid fluid obtained at high speed. (6) Use the S.N.F. in precipitin test with type sera.

**B10-724 TEST.**—(1) Set up a series of T.T. containing undiluted type sera. (2) Add antigen (**B10-721**) to form a layer above the serum. (3) Keep a few min. at 50 to 55°. (4) Examine for the formation of a ring precipitate at the interface between the two fluids. (5) Determine the 'type' of the antigen from the result.

**B10-725 TEST.**—(1) Mix clear test pneumonia serum or urine in precipitin test with type sera undiluted and diluted 1-2. (2) Examine immediately and after 1 hr. at R.T. (3) Determine the type from the precipitin results.

**B10-73 B. MALLEI: B10-731 ANTIGEN.**—(1) Make a suspension with 0.85 S.S.S. of a 3d. glycerin agar culture. (2) Keep 2d. at 37°C. (3) Filter through a Berkefeld or Chamberland candle.

**B10-732 ANTIGEN.**—(1) Prepare a sol. of a 48-hr. agar growth in 10 c.c. 8 per cent antiiformin. (2) Neutralize by careful addition of 5 per cent sulphuric acid. (3) Filter through paper and through a Berkefeld candle.

**B10-733 TEST.**—(1) Place undiluted test serum in the T.T. (2) Add an equal vol. of dilution of antigen (**B10-731**) without mixing the fluids. (3) Keep 30 min. at 37°C. (4) Examine for the occurrence of precipitation. (5) Place overnight in the ice chest. (6) Note the T.T. in which precipitation has taken place and its amount.

**B10-734.**—(1) Place 1 c.c. filtrate in a small T.T. (2) Add with a capillary pipette, 1 c.c. test serum to form a layer below the antigen sol. (**B10-732**). (3) Observe the formation<sup>1</sup> of a ring precipitate at the interface between the 2 fluids.

**Notes.** <sup>1</sup>Should occur immediately or at least within 15 min.

#### **B10-74. TUBERCULOSIS.<sup>1</sup>**

**Notes.** <sup>1</sup>The test has not proved satisfactory.

**B10-75 B. ANTHRACIS: B10-751 ANTIGEN.**—(1) Use spleen pulp for test. (2) Triturate in a mortar. (3) Extract 2 grm. pulp 5 hr. at R.T. with 10 c.c. chloroform. (4) Pipette off the S.N.F. (5) Extract the deposit with 7 or 8 c.c. 0.85 S.S.S. 2 hr. at R.T. (6) Filter clear through paper.

**B10-752 ANTIGEN** <sup>1</sup>—(1) Use spleen pulp<sup>2</sup> for test. (2) Make a suspension in 7 to 8 vol. of 0.85 S.S.S. (3) Boil for some time. (4) Filter through paper or a Berkefeld candle. (5) Test the filtrate when cold by the precipitin test against specific anthrax serum.

**Notes.** <sup>1</sup>Thermoprecipitin test. <sup>2</sup>Blood will serve the purpose

**B10-753 ANTIGEN.**—(1) Boil the test blood or tissue 3 min. in 8 vol. 0·85 S.S.S. containing 1-1000 acetic acid. (2) Allow to cool. (3) Filter through paper or asbestos. (4) Use in thermoprecipitin test.

**B10-754 TEST.**—(1) Place some drops of clear antigen sol. **B10-751, B10-752** in a small T.T. (2) Pass the anti-serum with a capillary pipette to form a layer under the antigen. (3) Watch for the formation of a ring precipitate<sup>1</sup> at the interface of the 2 fluids.

**Notes.**—<sup>1</sup> This precipitate should appear immediately with anthrax material, and not within 15 min. if the material is not anthrax.

**B10-76 MENINGOCOCCUS.**—(1) Centrifuge cerebrospinal fluid. (2) Add 1 drop of anti-serum to 75 drops of the cerebrospinal fluid. (3) Leave 10 hr. at 52°C. (4) Watch for the formation of a precipitate.

**B10-77 B. DIPHTHERIÆ ANTITOXIN STANDARDIZATION : B10-771 ANTIGEN.**—(1) Saturate filtrate of cultures of *B. diphtheriæ* with sod. sulphate. (2) Dry the precipitate *in vacuo* over sulphuric acid or calc. chloride. (3) Prepare<sup>1</sup>:—dried precipitate 0·8; D.W. 10. (4) Prepare :—toxin sol. 1; 10 per cent neutral melted gelatin 1. (5) Distribute in T.T. in quantities of 1 c.c. (6) Allow to set.

**Notes.**—<sup>1</sup> The resulting fluid should kill a large G.P. subcutaneously in a dose of 1-800th c.c.

**B10-772 TEST.**—(1) Add to the T.T. containing toxin gelatin mixture 1 c.c. antitoxin in dilutions 1-20, 50.....200. (2) Keep 2 hr. at R.T. (3) Read.<sup>1</sup>

**Notes.**—<sup>1</sup> A bluish white ring precipitate appears above the interface between gelatin and serum.

**B10-78 B. TETANI ANTITOXIN STANDARDIZATION.**—(1) Proceed as in the case of *B. diphtheriæ* (**B10-77**).

**B10-8 PRECIPITIN ACTION : B10-81 ECHINOCOCCUS.**—(1) Set up a series of 2 T.T. containing 1 vol. echinococcus cyst fluid. (2) Introduce 1 vol. of test serum beneath the antigen fluid with a pipette. (3) Keep 1 hr. at 40 to 42°C. (4) Examine for a ring precipitate at the interface of the 2 fluids.

**B10-82 BLOOD AND SERUM STAINS : B10-821 ANTIGEN.**—(1) Extract, without shaking, pieces of stained cloth<sup>1</sup> paper etc., with 0·85 S.S.S. (2) Extract at the same time an unstained piece of paper and cloth. (3) Continue the extraction with 0·85 S.S.S. until a sufficient quantity<sup>1</sup>

of the suspected material has been dissolved out. (1) Filter the extract if turbid through hard filter paper. (5) Centrifugalize, if necessary, to clear.

**Notes.**—<sup>1</sup>If the stain material cannot be immersed, the encrustation may be scraped off and extracted with 0·85 S.S.S. <sup>2</sup>The length of time of the extraction depends on the solubility of the test material. If the extraction is to be kept up for more than an hour it should be carried out in the ice chest, and a little chloroform added as preservative. Old specimens may have to remain soaking for 24 hr. or longer. The passage of protein into sol. is demonstrated, if bubbles made by blowing into the extracting fluid tend to persist foam test. Another test for sol. is the appearance of cloudiness on heating 2 c.c. of extract and adding 1 drop of 25 per cent nitric acid. Only a dilute sol. 1-1000 of protein is required for the test. Extracts of blood stain usually require dilution to this strength. The dilution is taken to be 1-1000, when the nitric acid heat test only just causes a faint opalescence. Comparative foam tests, known blood dilutions being used as the standard, also help to fix the strength of an extract. A 1-1000 dilution of blood is practically colourless by transmitted light. The extracts should be water clear. If they are turbid, they must be rendered clear by filtration or centrifugalization. The extract must not be either strongly acid or alkaline to litmus. In some cases it is wise to tease the material on which the suspected stain is found, and single threads in the substance may show blood which has been washed away from the bulk of the material.

**B10·822 ANTIGEN.**—(1) Use antigen **A3·418** or **A3·419**.

**B10·823 ANTI-SERUM STANDARDIZATION.**—(1) Set up a series of small T.T. each containing 0·5 c.c. 1-500, 1000, 1500, 2000..... antigenic serum. (2) Introduce 0·1 c.c. anti-serum by means of a capillary pipette to the bottom of each T.T. (3) Examine the T.T. for the occurrence of a ring precipitate. (4) Determine<sup>1</sup> the highest dilution of the antigenic serum which gives a ring precipitate with the anti-serum within 15 min.

**Notes.**—<sup>1</sup> If the dilution of antigen serum is at least 1-1000, the anti-serum is sufficiently potent for the test. When fowl anti-sera are used the salt sol. used should be 1·8 per cent to avoid non-specific reactions. The anti-serum should give no precipitate with other antigens or only in very low dilutions of the latter. A somewhat higher dilution may be permissible in the case of group antigen. No precipitate should be given with 0·85 S.S.S. The anti-serum should be perfectly clear.

**B10·824 TEST.**—(1) Prove the stain to be blood.<sup>1</sup> (2) Set up a first row of 6 small T.T. containing 0·1 c.c. anti-human rabbit serum. (3) Add to each T.T. in order 1·5 c.c. extracts of known human blood stain, known ox blood stain, known horse blood stain, known sheep blood stain, known pig blood stain, and the test blood stain. (4) Set up a second row of 4 small T.T. containing in order 0·1 c.c. normal rabbit serum, anti-ox rabbit serum, anti-sheep rabbit serum, and anti-pig rabbit serum. (5) Add to each T.T. of the second row 0·5 c.c. extract of tes

blood stain. (6) Keep at R. T. (7) Examine after 15 min.<sup>2</sup> and after 60 min.

**Notes.**—<sup>1</sup>The precipitin reaction reveals the presence not merely of blood but of any specific albuminous substance, such as mucus, pus, semen, milk or albuminous urine. The necessity then of proving the test stain to be due to blood is obvious. <sup>2</sup>Any precipitate that develops later than 20 min. is not admitted as having forensic significance.

**B10'83 BLOOD SERUM.—10'831.**—(1) Set up a series of small T.T. containing 2 c.c. of increasing dilutions of serum antigen.<sup>1</sup> (2) Allow 0.1 c.c. anti-serum<sup>2</sup> slowly to run down the side of each T.T. (3) Keep at R.T. (4) Read at intervals of 5 min. (5) Set up controls.

**Notes.**—<sup>1</sup>In the case of a blood stain the place of the serum antigen would be taken by an extract of blood stain in 0.85 S.S.S. <sup>2</sup>In forensic work the anti-serum must be such that 1 part added to 20 parts of 1:1000 serum antigen causes at the point of contact a cloudiness to appear immediately or at least within 1 or 2 min.

**B10'832.**—(1) Prepare antigen serum 1 : 10 per cent neutral gelatin in 0.85 S.S.S., 1. (2) Distribute in quantities of 1 c.c. in T.T. (3) Add to the gelatin antigen mixture 1 c.c. anti-serum in a series of dilutions. (4) Observe the precipitate above the interface between antigen and anti-serum.

**B10'84 MEAT : B10'841 ANTIGEN.**—(1) Remove test material for examination from the deeper parts of the specimen to be examined with a sterile knife. (2) Mince finely. (3) Weigh out a quantity of the minced material. (4) Cover<sup>1</sup> with 2 c.c. 0.85 S.S.S. per grm. (5) Keep 6 hr. at R.T. or overnight in the ice chest, with occasional shaking. (6) Test by foam test (**B10'8212**) for sufficiency of extraction. (7) Filter through a hard filter paper<sup>2</sup> wetted with 0.85 S.S.S. (8) Determine the dilution required to give sol. of protein of about 1:500, 1:1000 and 1:1500 by the heat and nitric acid test (**B10'8212**). (9) Test the reaction with litmus paper, and, if acid, neutralize very carefully with N-10 sod. hydroxide. (10) Prepare extracts of the same strength from known meat material, for comparison as controls with the test material.

**Notes.**—<sup>1</sup>A preliminary treatment by extraction for 18 hr. with a mixture of equal vol. of chloroform and ether, before proceeding to the extraction of the residue with 0.85 S.S.S. may be necessary. <sup>2</sup>If the filtrate be not perfectly clear, and especially if the test material is fat, or salt, it may be necessary to pass the filtrate through a Berkefeld candle.

**B10'842 ANTI-SERUM.**—(1) Prepare the various testing anti-sera by inoculation of rabbits intravenously with the serum<sup>1</sup> of the suitable species of animal.

**Notes.**—<sup>1</sup>It is not necessary to immunize with the meat extract itself.

**B10·843 TEST.**<sup>1</sup>—(1) Set up 4 small T.T. containing 2 c.c. 1-500, 1,000, 5,000, and 500, respectively, of test antigen sol. (**B10·841**). (2) Add to each, without any shaking, 0·1 c.c. of an anti-serum, such as horse or other species, according to the adulteration suspected. (3) Set up 4 control T.T. Nos. 1 to 3 containing 2 c.c. 1-500 horse, pig and ox extract, respectively, No. 4 containing 2 c.c. 0·85 S.S.S. (4) Add to each of the 4 control T.T. 0·1 c.c. of the anti-serum already used. (5) Read at R.T. (6) Examine for a ring precipitate at the interface between the two fluids.

**Notes.**—<sup>1</sup> The test is mainly used for the detection of adulteration in sausage meat.

**B10·9 WASSERMANN REACTION.**<sup>1</sup>—V. also **B9·8**.

**B10·91 : B10·911 GLASSWARE.** (1) Place T.T. and pipettes after thorough washing to soak in water for 3 hr. (2) Wash again in water. (3) Place to soak in soda sol. (4) Wash in weak hydrochloric acid followed by water, until the washings show no acid reaction. (5) Place to soak in a considerable vol. of D.W. (6) Allow to drain. (7) Keep 30 min. at 150°C. dry heat.

**B10·912 ERYTHROCYTES.** **B 9·711.v.**

**B10·913 HÆMOLYTIC SERUM STANDARDIZATION.**<sup>1</sup>—(1) Set up 9 small T.T. containing, respectively, 0·25 c.c. 1-2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000 inactivated rabbit anti-sheep hæmolytic serum. (2) Add 0·5 c.c. 1-4 dilution 5 per cent washed erythrocyte suspension. (3) Add to each T.T. 0·25 c.c. 1-10 fresh G.P. complementing serum. (4) Note the T.T. containing the greatest dilution of hæmolytic serum in which there is complete hæmolysis—the Minimum Hæmolytic Dilution (M.H.D.).

**Notes.**—<sup>1</sup> V. also **B9·713**. The standardization need not be done oftener than every 3 months. The titre should not be less than 1-1000.

**B10·914 SENSITIZED ERYTHROCYTE SUSPENSION.** (1) Prepare a dilution of the inactivated hæmolytic serum which is 10 times as strong as that representing the M.H.D. (2) Add this dilution in equal vol. to a 5 per cent washed erythrocyte suspension (**B10·917**).

**B10·915 COMPLEMENT.**—V. **B9·714**.

**B10·916 COMPLEMENT STANDARDIZATION.**<sup>1</sup>—(1) Set up 2 rows of 8 T.T. each. (2) Prepare dilutions 1-20, 25, 30, 35, 40, 50, 60, 80 of fresh complementing G.P. serum with 0·85 S.S.S. (3) Add 0·25 c.c. 1-80 dilution of serum to No. 1 T.T. of each row, 0·25 c.c. 1-60 dilution to No. 2 T.T. of each row, and so on down to 1-20 dilution. (4) Add

0.5 c.c. 0.85 S.S.S. to each T.T. of the first row and 0.25 c.c. 0.85 S.S.S. to each T.T. of the second row. (5) Add 0.25 c.c. 1:15 heart extract cholesterol antigen (**B10.917**) to each T.T. of the second row. (6) Keep all the T.T. 30 min. at R.T. (7) Keep 30 min. at 37°C. (8) Add to each T.T. 0.25 c.c. 2.5 per cent sensitized erythrocyte suspension. (9) Keep 30 min. at 37°C. shaking at the beginning and every 10 min. after. (10) Note the T.T. in the first row containing the greatest dilution of complementing serum in which there is complete hemolysis. Minimum hemolytic dilution (M.H.D.). (11) Note also the T.T. in the second row containing the greatest dilution of serum in which there is complete hemolysis.

**Notes.** <sup>1</sup> T. also **B9.715**. Standardization has to be carried out on each occasion of test. This T.T. should be the one containing double the dose of complement of that T.T. of the first row which gives the M.H.D. If this T.T. does not show complete hemolysis then the complementing serum should be rejected. A serum with minimum hemolytic dilution less than 1:30 should be rejected.

**B10.917 ANTIGEN.** (1) Mince finely fat free ox heart. (2) Grind up the heart material with fine sand. (3) Add by degrees 9 c.c. abs. alc. per grm. (4) Shake frequently for 3 hr. (5) Keep 48 hr. at R.T. (6) Filter through filter paper which has been washed with ether and allowed to dry. (7) Keep the filtrate 7d. at R.T. (8) Keep for use the S.N.F. in the ice chest. (9) Precipitate and dissolve with gentle heat:—cholesterol 1; abs. alc. 100. (10) Prepare at the time of actual test:—heart extract 3; alc. cholesterol sol. 2. (11) Dilute this mixture 1:15 by the rapid addition of 0.85 S.S.S.

**B10.918 TEST.**<sup>1</sup>—(1) Set out 3 rows,<sup>2</sup> of small T.T. (2) Add 0.25 c.c. 1:5 inactivated known positive serum to No. 1 T.T. of the first row. (3) Add 0.25 c.c. 1:5 inactivated test sera to each of Nos. 2, 3, 4, . . . T.T. of the first row. (4) Add 0.25 c.c. 1:5 inactivated known normal serum to the last T.T. of the first row. (5) Make a dilution of the complementing serum which is 3 times as strong as that representing the M.H.D. (6) Add 0.25 c.c. of this dilution to each T.T. of the first and third rows. (7) Make a dilution of the complementing serum which is 5 times as strong as that representing the M.H.D. (8) Add 0.25 c.c. of this dilution to each T.T. of the second row. (9) Add 0.25 c.c. 1:15 heart extract cholesterol antigen to each T.T. of the first and second rows and 0.25 c.c. 0.85 S.S.S. to each T.T. of the third row. (10) Keep 30 min. at R.T. (11) Keep 30 min. at 37°C. (12) Add to each T.T. of all three rows 0.25 c.c. sensitized erythrocyte suspension. (13) Shake to mix. (14) Keep at 37°C.

(15) Shake after 15 min. to mix. (16) Watch the third row T.T. and note for re-test<sup>3</sup> if in any case the hæmolysis in any T.T. lags behind that of others in the row. (17) Watch the last T.T. in each row for the occurrence of hæmolysis. (18) Remove the T.T. from the water bath 10 min. after hæmolysis is complete in the last T.T. of the first row. (19) Keep 1 hr. in a water bath containing ice. (20) Read the results.

**Notes.**—<sup>1</sup> Other methods of test involve quantitative variations in the quantities of heart extract cholesterin antigen, or in the quantities of the sera, or in the quantities of complement. <sup>2</sup>A fourth row of control T.T. may be used to test whether any deterioration of complement dilution has occurred on standing. <sup>3</sup>Where any third row T.T. shows lag, the test should be repeated for the serum in question, unless there is hæmolysis in the corresponding T.T. of the first row showing that the reaction is negative.

**B10'92 : B10'921 ANTIGEN.**—V' A3'614 A6'15 alc. soluble acetone-insoluble antigen.

**B10'922 ANTIGEN.**—(1) Grind up finely the liver of a congenitally syphilitic child. (2) Spread thinly in Petri dishes. (3) Dry *in vacuo* over sulphuric acid or calc. chloride. (4) Prepare :—antigen powder 20 grm. ; alc. 100 c.c. (5) Keep 18d. at R.T. (6) Add 25 c.c. more alc. (7) Use in test.

**B10'923 ANTIGEN.**—(1) Grind up in a mortar with broken glass 20 grm. fat free bullock's heart. (2) Add 5 c.c. abs. alc., and 0·5 c.c. 1 per cent cholesterin in abs. alc., for each grm. of heart. (3) Place the entire contents of the mortar in a sterile flask and apply a rubber cap. (4) Keep 24 hr. at 37C. (5) Shake up. (6) Filter through 2 thicknesses of filter paper into small bottles. (7) Allow to stand<sup>1</sup> 48 hr. (8) Use the S.N.F. as antigen.

**Notes.**—<sup>1</sup>A fine flocculent precipitate settles to the bottom.

**B10'924 ANTIGEN STANDARDIZATION,<sup>1</sup> ANTICOMPLEMENTARY.**—(1) Dilute by slow addition the antigen extract (A3'614) 1-10 with 0·85 S.S.S. (2) Set up a series of 8 small T.T. containing 0·2, 0·4, 0·6, 0·8, 1, 1·2, 1·5 and 2 c.c. of 1-10 antigen. (3) Add 1 c.c. 1-20 complementing serum. (4) Bring the total vol. in the T.T. up to 3 c.c. (5) Set up controls.<sup>2</sup> (6) Shake gently to mix. (7) Keep 1 hr. at 37C. (8) Add to each T.T. 1 c.c. 2·5 per cent washed sheep erythrocytes (B9'711) and 2 M.H.D. (B9'713, B10'913) of hæmolytic serum. (9) Shake gently to mix. (10) Keep 90 min. at 37C. (11) Make the first reading. (12) Keep overnight in the ice chest. (13) Determine the

anti-complementary unit.<sup>3</sup> (14) Use the 1-10 antigen in test in an amount equal to 1-4th the anti-complementary unit.

**Notes.**—<sup>1</sup> V. also **B9-716**. <sup>2</sup>No. 1 control T.T. containing erythrocyte suspension, complement, and hæmolytic serum; No. 2 control containing erythrocyte suspension 0.2 c.c. inactivated test serum, complement and hæmolytic serum, to show that the test serum itself is not anti-complementary. <sup>3</sup>The least amount of antigen which shows commencing inhibition of hæmolysis = the anti-complementary unit.

**B10-925 ANTIGEN. STANDARDIZATION<sup>1</sup> ANTIGENIC POWER.**—(1) Conduct this standardization in a similar manner to the anti-complementary (**B10-924**) but with the addition of 0.2 c.c. inactivated known positive syphilitic serum to the components of the mixtures. (2) Determine that amount of antigen which just shows complete inhibition of hæmolysis. (3) Use 3 times this amount in the test if this quantity is less than 1-4th the anti-complementary unit (**B10-9243**).

**B10-926 TEST.**—(1) Set up 8 small T.T. (2) Add to Nos. 1 to 7 T.T. the appropriate<sup>1</sup> dose of antigen. (3) Add to Nos. 1 to 8 T.T. 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, and 2 c.c. inactivated 1-10 dilution of test serum, or the same quantities of cerebro-spinal fluid fresh and undiluted. (4) Add 1 c.c. 1-20 fresh G.P. complementing serum to each T.T. (5) Bring the total vol. in the T.T. up to 3 c.c. (6) Set up controls of known normal and positive sera, of the complementing serum, of the antigen extract, and of the erythrocyte suspension. (7) Shake gently to mix. (8) Keep all the T.T. 1 hr. at 37°C. (9) Add 2 M.H.D. (**B9-713**, **B10-913**) of inactivated hæmolytic serum and 1 c.c. 2.5 per cent suspension washed sheep erythrocytes to all T.T. except that representing the erythrocyte suspension control. (10) Keep 60 min. or longer at 37°C. according to the results given by the control T.T.

**Notes.**—<sup>1</sup> V. **B10-924** and **B10-925**. The quantity of antigen which is 3 times the antigenic unit and at the same time not more than 1-4th the anti-complementary unit is an appropriate amount.

**B10-927 TEST.**—(1) Set up 8 small T.T. containing 0.1 c.c. inactivated test serum. (2) Add to Nos. 1 to 6 T.T. the appropriate<sup>1</sup> dose of antigen extract and 2, 3, 4, 5, 6, and 8 M.H.D. (**B9-715**) of complementing serum, respectively. (3) Add 1 M.H.D. (**B9-713**, **B10-913**) of hæmolytic serum and 0.5 c.c. washed sheep erythrocytes to each T.T. (4) Keep 1 hr. at 37°C. (5) Read. (6) Place in the ice chest overnight. (7) Read again.

**Notes.**—<sup>1</sup> V. **B10-924** and **B10-925**. The quantity of antigen which is 3 times the antigenic unit and at the same time not more than 1-4th the anti-complementary unit is an appropriate amount.

**B10'93.**—(1) Set up 2 small T.T. containing 0·1 c.c.<sup>1</sup> test serum which must not be more than 18 hr. old. (2) Add to No. 1 T.T. 0·1 c.c. antigen.<sup>2</sup> (3) Make up the total vol. in each T.T. to 1·3 c.c. with 0·85 S.S.S. (4) Keep 30 min. at 37°C. (5) Add to both T.T. 0·1 c.c. 10 per cent washed human erythrocytes and 1 M.H.D. (**B9'713, B10'913**) hæmolytic serum in a vol. of 0·1 c.c. (6) Shake carefully to mix. (7) Keep 30 min. at 37°C., shaking the T.T. at 10 min. intervals. (8) Keep 30 min. at R.T. (9) Read the result. (10) Add another M.H.D. hæmolytic serum to both T.T., if hæmolysis is incomplete in the control T.T. and keep as before 30 min. at 37°C. (11) Repeat the test from the beginning with the dilution of 0·1 c.c. fresh known normal human serum as complement, if the hæmolysis should again be incomplete. (12) Set up controls throughout of pairs of T.T. for known positive and known normal sera.

**Notes.** <sup>1</sup>If inactivated serum is used in the test instead of fresh active serum, the dose is 0·2 c.c. If cerebro-spinal fluid is used, the dose is 0·5 c.c. In both these cases it is necessary to add 0·1 c.c. known normal fresh human serum as complement. <sup>2</sup>A 1·10 suspension in 0·85 S.S.S. of the acetone-insoluble fraction of tissue lipid (**A3'614, A3'615**) dissolved in pure methyl alc. in the proportion of 3 per cent.

**B10'94**—(1) Set up 14 small T.T. containing 0·1 c.c. fresh unheated test serum. (2) Add to Nos. 1 to 10 T.T. in order 1, 0·9, 0·8, 0·7, 0·6, 0·5, 0·4, 0·3, 0·2 and 0·1 c.c. of 0·85 S.S.S. (3) Add to Nos. 1 to 10 T.T. in the same order 0·1, 0·2, 0·3, 0·4, 0·5, 0·6, 0·7, 0·8, 0·9 and 1 c.c. 5 per cent suspension sheep's erythrocytes. (4) Keep Nos. 1 to 10 T.T. 30 min. at 37°C. (5) Read the hæmolytic index.<sup>1</sup> (6) Add to Nos. 11 to 13 T.T. the proper amount <sup>2</sup> of sheep erythrocyte suspension together with increasing strengths of antigen.<sup>3</sup> (7) Add to No. 14 T.T. sheep erythrocytes, without antigen. (8) Keep 30 min. at 37°C. (9) Read.

**Notes.**—<sup>1</sup>The T.T. which last shows complete hæmolysis gives the hæmolytic index in terms of amount of erythrocyte suspension dissolved. <sup>2</sup>Dependent on the hæmolytic index. If the index is 0·1 to 0·4 c.c. add 0·1 c.c. sheep erythrocyte suspension; 0·5 to 0·7 c.c. add 0·15 sheep erythrocyte suspension; 1 to 1·5 c.c. add 0·25 c.c.; 1·8 to 2 c.c. add 0·35 c.c. <sup>3</sup>If the amount of complement or natural anti-sheep hæmolysin is very low, add the proper amount of fresh G.P. complementing serum or rabbit anti-sheep serum.

**B10'95: B10'951 ANTIGEN.**—(1) Use **A 3'613**.

**B10'952 TEST.**—(1) Prepare:—test or control fresh serum 1; antigen 4. (2) Keep 1 hr. at 37°C. (3) Add 5 per cent sheep erythrocyte suspension 1. (4) Mix. (5) Keep 1 hr. at 37°C. (6) Read results of action of test and of control sera.

**B10'96.**—(1) Add 1 c.c. test blood to 3 c.c. ammon. oxalate sol. and mix immediately. (2) Set up 4 T.T. containing 17, 16, 15, and 18 drops oxalated blood. (3) Add to Nos. 1, 2 and 3 T.T. 1, 2 and 3 drops antigen. (4) Keep 15 min. at 37°C. (5) Add 1 drop inactivated anti-human rabbit hemolytic serum.<sup>1</sup> (6) Keep at 37°C. until the controls show hemolysis. (7) Read.

**Notes.**—<sup>1</sup>The test may be rendered more delicate by standardization of the agglutinating serum.

## INDEX.

**ABDERHALDEN.**—TEST B10·1.

**ABRIN.**—ANTITOXIN B8·97

**ABSORPTION.**—MENINGOCOCCUS B8·32—TEST B8·31—TEST ANTIGEN  
B8·311—TEST OF ANTIGEN B8·312—TEST OF SERUM,

**B. ÆROGENES CAPSULATUS.**—V. B. WELCHII.

**AGAR.**—ANAPHYLAXIA B8·4411.

**AGGLUTINABILITY.**—ANTIGEN STANDARDIZATION B8·133

**AGGLUTINATION.**—AND SEDIMENTATION B8·1221—B. ANTHRACIS B8·24  
—ANTIGEN B8·1111, B8·121—CELL METHOD B8·152—  
DREYER'S METHOD B8·13—DYSENTERY ORGANISMS  
B8·1128, B8·1321—END POINT B8·1221—ERYTHROCYTES  
B8·33—GROUP B8·1323—HANGING DROP B8·151—  
INAGGLUTINABLE STRAINS B8·17—INOCULATED  
PERSONS B8·1121—MENINGOCOCCUS B8·1128, B8·1321  
—MICROSCOPIC B8·15—B. PESTIS B88·1211—PRIMARY  
B8·1323—REACTION B8·1—SECONDARY B8·1323—SLIDE  
METHOD B8·14—SPONTANEOUS B8·1211—STANDARD  
B8·1333—THREAD REACTION B8·153—B. TUBER.  
CULOSIS B8·1211—TYPHOID ORGANISMS B8·1128—  
UNIT B8·1124, B8·1335, B8·1344—UNIT DREYER  
B8·1935—WRIGHT'S METHOD B8·12.

**ALBUMIN.**—BIURET TEST B10·115—IMPERMEABILITY DIALYZER B10·115.

**ALLERGIC.**—ACTION B8·4.

**AMBOCEPTOR.**—SERUM V. HÆMOLYTIC SERUM.

**ANAPHYLACTIC.**—ACTION B8·4, B8·5.—SHOCK B8·41.

**ANAPHYLACTIC SHOCK.**—CAT B8·417—DOG B8·418, B8·444—EGG WHITE B8·413  
—ERYTHROCYTES B9·7121—G.P. B8·411 TO  
B8·414, B8·4431, B8·4451—MILK B8·412—RABBIT  
B8·415, B8·416, B8·4431, B8·4451—RAT B8·4431,  
B8·4451—SERUM B8·411, B8·414 TO B8·418—  
WITHOUT ANTI-SERUM B8·44—WITHOUT  
SENSITIZATION B8·44.

**ANAPHYLAXIA.**—AGAR B8·4411, B8·445—BACTERIAL B8·4251, B8·43, B8·4411,  
B8·442—CONJUNCTIVAL B8·46, B8·477, B8·491, B8·531—  
CUTANEOUS B8·45, B8·47, B8·481, B8·492, B8·511, B8·52,  
B8·533, B8·541, B8·915—NASAL B8·478—PASSIVE B8·42  
—PEPTONE B8·4411, B8·443, B8·444—POLLEN B8·53—  
URETHRAL B8·479—VAGINAL B8·479—WITHOUT ANTI-  
SERUM B8·44.—WITHOUT SENSITIZATION B8·44.

**B. ANTHRACIS.**—AGGLUTINATION B8-24—ANTIEMETOXIN B8-75—ANTIGEN B10-751 to B10-753—COMPLEMENT FIXATION B9-731—PRECIPITIN TEST B10-754.

**ANTIANAPHYLAXIA.**—V. DESENSITIZATION.

**ANTIBACTERIAL ACTION.**—SERUM B8-7.

**ANTI-COMPLEMENTARY.**—STANDARDIZATION ANTIGEN B10-924—UNIT B10-9243.

**ANTIENDOTOXIC ACTION.**—B. ANTHRACIS B8-75—V. CHOLERÆ B8-71—B. DYSENTERIÆ SHIGA B8-72—MENINGOCOCCUS B8-74—SERUM B8-7—STANDARDIZATION B8-7—B. TYPHOSUS B8-73—UNIT B8-724 B8-731.

**ANTIGEN.**—ABSORPTION TEST B8-311—ACUTE POLIOMYELITIS B9-744—AGGLUTINATION B8-111, B8-121, B8-131, B8-311—B. ANTHRACIS B8-24, B10-751 to B10-753—BACTERIAL B10-511, B10-512 B10-711 to B10-714—BACTERIAL HÆMOLYSIN B8-842—BACTERIOLYTIC TEST B9-411, B9-412—BLOOD STAIN B10-821—CANCER B9-811, B9-812, B10-513, B10-514—CHOLESTERINIZED B10-3312, B10-391—DANGEROUS ORGANISMS B10-6112—DILUTION B9-321—B. DIPHTHERIÆ B10-771—DOURINE B9-741—ECHINOCOCCUS B9-743—ERYTHROCYTE B9-711, B9-721 to B9-726—FLESH B9-761, B10-841—FORMALINIZED B8-1111, B8-131—GONOCOCCUS B8-1711, B9-732 to B9-734—HYDATID CYST B9-743—SP. ICTEROHÆMORRHAGIÆ B8-261—INAGGLUTINABLE B8-17—B. MALLEI B8-23, B8-736, B8-737, B10-731, B10-732—MEAT B9-761, B10-841—MEIOSTAGMIN TEST B10-51, B10-61—MENINGOCOCCUS B8-18, B8-741, B9-735, B10-76—OPSONIC ACTION B10-61—SP. PALLIDA B8-25, B8-541—PNEUMOCOCCUS B8-1711, B8-21, B10-613, B10-721 to B10-723—PNEUMOCOCCUS SPUTUM B10-721 to B10-723—POLLEN B8-53—PRESERVATION B8-111—SACHS GEORGI TEST B10-391—SAUSAGE MEAT B9-762—SPOROTHRIX B8-28—STANDARDIZATION B8-132, B8-133, B9-322, B9-716, B10-52, B10-924, B10-925—STREPTOCOCCUS B8-19—SUSPENSION B8-111, B8-121, B8-131—SYNTHETIC CANCER B10-5141—SYPHILITIC B10-515, B10-922—B. TUBERCULOSIS B8-22, B9-738, B10-614—UNIT B9-7167—WASSERMANN TEST B10-921 to B10-923.

**ANTIGENIC POWER.**—STANDARDIZATION ANTIGEN B10-925.

**ANTIHEMOLYTIC ACTION.**—BACTERIAL HÆMOLYSIN B8-81—SERUM B8-8—STANDARDIZATION B8-8122.

**ANTITOXIC ACTION.**—ABRIN B8-97—B. BOTULINUS B8-93—CROTIN B8-9711—B. DIPHTHERIÆ B8-9—HAY FEVER B8-98—B. OEDEMATIENS B8-96—RICIN B8-9711—V. SEPTIQUE B8-95—SERUM B8-9—STANDARDIZATION B8-9, B10-77—STANDARDIZATION BEHRING B8-912—STANDARDIZATION EHRLICH B8-913—STANDARDIZATION REMER B8-914—STANDARDIZATION ROUX B8-911—B. TETANI B8-92—B. WELCHII B8-94.

**ANTITOXIN.**—V. ANTITOXIC ACTION.—UNIT DIPHTHERIA B8-9135—UNIT TETANUS B8-9214.

**ANTITRYPTIC ACTION.**—CALCIFIED MILK METHOD B9-14—CASEIN ACETIC METHOD B9-11—GELATIN LIQUEFACTION METHOD B9-12, B9-13—WRIGHT B9-14.

**ANTIVENIN ACTION.**—COBRA VENOM B9-2—SNAKE VENOM B9-2.

**BACTERIA.**—ANAPHYLAXIA B8-4251 B8-43, B8-4411, B8-442—COMPLEMENT FIXATION B9-73—PRECIPITIN ACTION B10-71—SPONTANEOUSLY AGGLUTINABLE B8-1211, B8-1312.

**BACTERICIDAL ACTION.**—COLONY COUNT METHOD B9-31—COMPLETE STERILIZATION METHOD B9-32.

**BACTERIOLYTIC ACTION.**—COMPLEMENT B9-61—TEST ANTIGEN B9-411, B9-412—TEST CHOLERA B9-4111, B9-4131, B9-42—TEST SERUM B9-413.

**BEHRING.**—STANDARDIZATION ANTITOXIN B8-912.

**BIURET TEST.**—ALBUMIN B10-115.

**BLOOD.**—COMPLEMENT FIXATION B9-75—CULTURE B8-1121—GROUPS B8-3321, B10-4221—OXALATED B10-96—TRANSFUSION TESTS B8-33, B10-42.

**BLOOD SERUM REACTIONS.**—ABDERHALDEN TEST B10-1—ABSORPTION B8-31—AGGLUTINATION B8-1—ANAPHYLACTIC B8-4, B8-5—ANTIANAPHYLACTIC B8-6—ANTIBACTERIAL B8-7—ANTI-ENDOTOXIC B8-7—ANTIHÆMOLYTIC B8-8—ANTITOXIC B8-9—ANTITRYPTIC B9-1—ANTIVENIN B9-2—BACTERICIDAL ACTION B9-3—BACTERIOLYTIC ACTION B9-4—COBRA VENOM REACTION B9-5—COMPLEMENT ACTION B9-6—COMPLEMENT FIXATION ACTION B9-7—CYTOLYTIC ACTION B9-8—DIGESTIVE ACTION B10-1—EPIPHANIN REACTION B10-2—FERMENT ACTION B10-1—FLOCCULATION REACTION B10-3—HÆMOLYTIC ACTION B10-4—MEIOSTAGMIN REACTION B10-5—OPSONIC ACTION B10-6—PRECIPITIN ACTION B10-7, B10-8—TRANSFUSION B8-33—TYPE, SEROLOGICAL B8-3261—WASSERMANN REACTION B10-1.

**BLOOD STAIN.**—ANTIGEN B1-821—ANTISERA STANDARDIZATION B9-751—COMPLEMENT FIXATION TEST B9-752—EXTRACT B10-821—PRECIPITIN TEST B10-824.

**B. BOTULINUS.**—ANTITOXIN B8-93.

**BROWN AND IYENGAR.**—TESTS SYPHILIS B9-522, B10-31.

**BRUCK TESTS.**—SYPHILITIC SERUM B10-32.

**BUTYRIC ACID.**—TEST SYPHILIS B10-371.

**CANCER.**—ANTIGEN B9-811, B9-812, B10-513—COBRA VENOM REACTION B8-53—CYTOLYTIC TEST B9-81—DIAGNOSIS B9-1161, B9-53, B9-81.

**CASEIN.**—ANTITRYPTIC METHOD B9-11—SOLUTION B9-112.

**CAT.**—ANAPHYLACTIC SHOCK B8417.

**CEREBRO-SPINAL MENINGITIS.**—INTRACUTANEOUS TEST B8411.

**V. CHOLERÆ.**—ANTIENDOTOXIN B871—BACTERIOLYTIC TEST B9011,  
B94131, B9412—PREIFFER PHENOMENON B9412.

**CHOLESTERIN.**—ANTIGEN B10331, B10391, B10917, B10921.

**COBRA VENOM.**—ANTIVENIN B92—PSYCHO REACTION B951—REACTION  
B95 REACTION—CANCER B953—REACTION—SYPHILIS  
B952.

**B. COLI.**—ANTIGEN—COMPLEMENT FIXATION B9731—OPSONIC ACTION  
B106111.

**COMPLEMENT.**—ACTION SERUM B96—BACTERIOLYTIC B961—END PLATE  
B961—FIXATION ACTION B97, B108—HEMOLYTIC B962,  
B9714—MIDDLE PIECE B961—MINIMUM HEMOLYTIC  
DOSE B962, B97154—PRESERVATION B9727 to B9729—  
PROCÜRAL B9714—STANDARDIZATION B9715, B10916—  
SPLITTING B961.

**COMPLEMENT FIXATION.**—ACTION B97, B108—ACUTE POLIOMYELITIS  
B9744—BACTERIA B973—BLOOD B975—BLOOD  
STAIN B9751—DOURINE B9741, B9742—  
ECHINOCOCCUS B9743—GONOCOCCUS B9732 to  
B9734—B. MALLEI B9736, B9737—MEAT B976—  
MENINGOCOCCUS B9735—SYPHILIS B109—TEST  
B9717—B. TUBERCULOSIS B9738, B9739.

**CONJUNCTIVAL TEST.**—HAY FEVER B8531—HAY FEVER ANTITOXIN  
B8532, B8981—MINIMUM REACTING DOSE  
ANTIGEN B85312—TUBERCULIN B84774—  
TUBERCULOSIS B84601, B8477—TYPHOID FEVER  
B8491.

**CROTIN.**—ANTITOXIN B89711.

**CUTANEOUS.**—ANAPHYLAXIA B845, B847, B8481, B845, B8433, B8541,  
B8915—TESTS B84501, B847.

**CUTANEOUS TEST.**—HAY FEVER B8533—V. PIRQUET B8471—SYPHILIS  
B8541—TYPHOID FEVER B4892—TUBERCULOSIS B847,  
B8471.

**CUTI-REACTION.**—V. ANAPHYLAXIA CUTANEOUS, AND CUTANEOUS TEST.

**CYTOLYTIC ACTION.**—CANCER SERUM B98112—SERUM B98.

**DESENSITIZATION.**—INTRAPERITONEAL B863—INTRAVENOUS B862.

ORAL B865—RECTAL B866—SUBCUTANEOUS B861,  
B8632—SURDURAL B864.

**DIALYZING SHELL.**—IMPERMEABILITY ALBUMIN B10115—PERMEABILITY  
PEPTONE B10116—PREPARATION B10114.

**DIGESTIVE ACTION**—SERUM B101.

**B. DIPHTHERIÆ.**—ANTIGEN B10771—ANTITOXIN B891—ANTIDOODEN TOXIN  
B89135—INTRACUTANEOUS TEST B847—PRESTIGE  
STANDARDIZATION B1077—TOXIN M.L.D. B89133.

**DOG.**—ANAPHYLACTIC SHOCK B8418, B9444.

**DOURINE.**—ANTIGEN B9741, B9742—COMPLEMENT FIXATION B9741,  
B9742.

- DREYER.**—AGGLUTINATION TEST B8-13—AGGLUTININ UNIT B8-1335
- DROP.**—PIPETTE MEASUREMENT B8-1124—UNIT VOLUME B8-1124.
- B. DYSENTERIÆ SHIGA.**—ANTIENDOTOXIN B8-72.
- DYSENTERY.**—ORGANISMS AGGLUTINATION B8-1128, B8-1321, B8-1332.
- ECHINOCOCCUS.**—ANTIGEN B9-743—COMPLEMENT FIXATION B9-743—PRE-CIPITIN TEST B10-81.
- EGG WHITE.**—ANAPHYLACTIC SHOCK B8-413.
- EHRlich.**—STANDARDIZATION ANTI-TOXIN B8-913.
- ENTERIC FEVER.**—V. TYPHOID FEVER.
- ERYTHROCYTE.**—AGGLUTINATION B8-33—ANAPHYLACTIC SHOCK B9-7121—ANTIGEN B9-711, B9-721 to B9-726—GROUPS B8-3321—HÆMOLYSIS B10-4—SUSPENSION B9-711, B9-721 to B9-726, B10-14—PRESERVATION B9-721 to B9-726.
- FERMENT ACTION.**—DIALYZING SHELL B10-114—SERUM B10-10001—SUBSTRATE, PREPARATION B10-113.
- FLEMING.**—WASSERMANN TEST B10-95.
- FLESH.**—ANTIGEN B9-761, B10-861—ANTISERUM B9-7612, B10-842—COMPLEMENT FIXATION B9-76—PRECIPITIN TEST B10-84.
- FLOCCULATION TEST.**—SYPHILITIC SERA B10-3.
- FOAM TEST.**—PROTEIN B10-8212.
- FOCAL.**—REACTION B8-4701.
- FORMALIN.**—ANTIGEN B8-1111, B8-241, B9-721, B9-723, B9-725
- GELATIN.**—ANTITRYPTIC METHOD B9-12, B9-13
- GLANDERS.**—SUBCUTANEOUS TEST B8-481.
- GLASSWARE.**—CLEANING B10-911.
- GONOCOCCUS**—AGGLUTINATION B8-1711—ANTIGEN B8-1711, B9-732 to B9-734, B10-6111—COMPLEMENT FIXATION B9-732 to B9-734—OPSONIC ACTION B10-6111.
- GROUP.**—AGGLUTININ B8-1323—BLOOD B8-3321—PRECIPITIN B10-8231.
- GUINEA PIG.**—ANAPHYLACTIC SHOCK B8-411 to B8-414, B8-4431, B8-4451—COMPLEMENT B9-714.
- HÆMOLYSIN.**—BACTERIAL B8-812.
- HÆMOLYTIC ACTION.**—COMPLEMENT B9-62—SERUM B10-4.
- HÆMOLYTIC SERUM.**—PRODUCTION B8-6221, B9-712—STANDARDIZATION B9-713, B10-41, B10-913.
- HANGING DROP.**—AGGLUTINATION B8-151.
- HAY FEVER.**—ANTITOXIN B8-98—ANTITOXIN TEST B8-532, B8-981—CONJUNCTIVAL TEST B8-531—CUTANEOUS TEST B8-533—MINIMUM REACTING DOSE ANTIGEN B8-5312.
- HECHT.**—WASSERMANN TEST B10-94.
- HERMANN AND PERUTZ TEST.**—SYPHILITIC SERUM B10-33.
- HIGH TITRE SERA.**—AGGLUTINATING V. AGGLUTINATION.
- HIRSCHFELD AND KLINGER TEST.**—SYPHILITIC SERUM B10-34.
- HORSE FLESH.**—DETECTION B9-7621.
- HYDATID CYST.**—COMPLEMENT FIXATION B9-743—PRECIPITIN TEST B10-81.
- SP. ICTEROHÆMORRHAGIÆ.**—AGGLUTINATION B8-26—ANTIGEN B8-261—COMPLEMENT FIXATION B9-7311.

**L. ICTEROIDES.**—AGGLUTINATION B8-27.

**INTRACUTANEOUS TEST.**—B. DIPHTHERIE B8-352—MENINGOCOCCUS  
B8-511—TUBERCULOSIS B8-473—TUBERCULOSIS  
ANIMAL B8-474—PROTEIN B8-451

**ISOAGGLUTININ.**—TEST B8-33, B10-4221.

**ISOHÆMOLYSIN.**—TEST B10-4221.

**ISOLATION.**—COLONIES B9-322

**JANSKY**—GROUP SERA ERYTHROCYTES B8-3321.

**KLAUSNER TEST.**—SYPHILITIC SERUM B10-35.

**L.**—DOSE B8-9137, B8-9214.

**Lo.**—DOSE B8-9137.

**LEUCOCYTE.**—SUSPENSION B10-62.

**MALIGNANT DISEASE.**—V. CANCER.

**B. MALLEI.**—AGGLUTINATION B8-23—8-23—ANTIGEN B8-23 B9-736, B9-737,  
B10-731, B10-732—COMPLEMENT FIXATION B9-736 B9-737—  
PRECIPITIN TEST B10-733, B10-734.

**MALLEIN.**—SUBCUTANEOUS ANIMAL TEST,—B8-481.

**MEAT.**—ANTIGEN B9-761, B10-841—ANTI-SERUM B9-7612, B10-842—COMPLE-  
MENT FIXATION B9-76—PRECIPITIN TEST B10-84.

**MEINICKE TEST.**—SYPHILITIC SERUM B10-36.

**MEIOSTAGMIN REACTION.**—SERUM B10-5.

**MENINGOCOCCUS.**—AGGLUTINATION B8-1128, B8-18—ANTIENDOTOXIN  
B8-74—ANTIGEN B8-18, B8-741, B9-735—COMPLEMENT  
FIXATION B9-735—INTRACUTANEOUS TEST B8-511—  
TYPE B8-321, B8-322.

**METHYLENE BLUE.**—ALKALINE B10-6421.

**MILK.**—ANAPHYLACTIC SHOCK B8-412—ANTITRYPTIC METHOD B9-14.

**MINIMUM HÆMOLYTIC DOSE.**—BACTERIAL HÆMOLYSIN B8-812—COMPLE-  
MENT B9-62, B9-7154, B10-916—HÆMOLYTIC  
SERUM B9-7131, B9-7134, B10-4113, B10-4122,  
B10-913.

**MINIMUM INHIBITING DOSE.**—ANTI-SERUM B9-5713.

**MINIMUM LETHAL DOSE.**—ANAPHYLAXIA B8-4211—DIPHTHERIA TOXIN  
B8-9133—SNAKE VENOM B9-222.

**MINIMUM REACTING DOSE.**—HAY FEVER ANTIGEN B8-5312.

**MORO.**—PERCUTANEOUS TEST B8-472.

**MOSS.**—GROUPS SERA ERYTHROCYTES B8-3321.

**MUCH.**—PSYCHOREACTION B9-511.

**NASAL TEST.**—TUBERCULOSIS B8-478.

**NINHYDRIN.**—SERUM TEST B10-1001—TEST PEPTONE B10-1101.

**NOGUCHI.**—TEST SYPHILITIC SERUM B10-37—WASSERMAN TEST B10-93

**B. ŒDEMATIENS.**—ANTITOXIN B8-296.

**OPHTHALMO REACTION.**—ANAPHYLACTIC B8-46—HAY FEVER B8-531—  
HAY FEVER ANTITOXIN B8-532, B8-581—  
MINIMUM REACTING DOSE B8-5312—  
TUBERCULIN B8-474—TUBERCULOSIS  
B8-4601, B8-477—TYPHOID FEVER B8-491.

**OPSONIC.**—INDEX B10-6418.

**OPSONIC ACTION.**—BACTERIA EASILY DISINTEGRATED B10-643—SERUM B10-6—B. TUBERCULOSIS B10-642.

**OXALATE.**—BLOOD B10-96.

**SP. PALLIDA.**—AGGLUTINATION B8-25—ANTIGEN B8-25, B8-541—ANTI-SERUM B8-251—INTRACUTANEOUS TEST B8-541.

**PEPTONE.**—ANAPHYLAXIA B8-4411, B8-443, B8-444—PERMEABILITY DIALYZER B10-115.

**PERCUTANEOUS TEST.**—MORO B8-472—TUBERCULOSIS B8-47, B8-472.

**B. PERFRINGENS.**—v. B. WELCHII.

**B. PESTIS.**—AGGLUTINATION B8-1211—COMPLEMENT FIXATION B9-7311.

**B. PERTUSIS.**—COMPLEMENT FIXATION B9-7311.

**PFEIFFER PHENOMENON.**—V. CHOLERÆ B9-42—TEST ANTIGEN B9-4211—TEST SERUM B9-4211.

**PHAGOCYtic ACTION.**—V. OPSONIC ACTION.

**v. PIRQUET.**—TEST B8-471.

**PNEUMOCOCCUS.**—AGGLUTINATION B8-1711, B8-21—ANTIGEN B8-1711, B8-21, B10-613—IDENTIFICATION OF TYPE B8-21—OPSONIC ACTION B10-613—SPUTUM ANTIGEN B10-721 TO B10-723.

**D. PNEUMONIÆ.**—v. PNEUMOCOCCUS.

**POLARIMETRIC.**—SERUM TEST B10-1001, B10-12.

**POLLEN.**—ANAPHYLAXIA B8-53—ANTIGEN UNIT B8-5312.

**PORGES TEST.**—SYPHILITIC SERUM B10-381.

**PORGES AND MEIER TEST.**—SYPHILITIC SERUM B10-382.

**PRECIPITIN ACTION.**—B. ANTHRACIS B10-75—BACTERIA B10-71—BLOOD SERUM B10-83—BLOOD STAIN B10-82—B. DIPHTHERIÆ B10-77—ECHINOCOCCUS B10-81—FLESH B10-84—B. MALLEI B10-73—MEAT B10-84—MENINGOCOCCUS B10-76—D. PNEUMONIÆ B10-72—SERUM STANDARDIZATION B10-823—B. TETANI B10-78—B. TUBERCULOSIS B10-74.

**PRESERVATION.**—ANTIGEN B8-1111—COMPLEMENT B9-727 TO B9-729—ERYTHROCYTES B9-721 TO B9-726.

**PRESERVATION REAGENTS.**—COMPLEMENT FIXATION TEST B9-72.

**PROTEIN.**—FOAM TEST B10-8212—HEAT-NITRIC ACID TEST B10-8212.

**PROTEOLYTIC ACTION.**—SERUM B10-1001.

**RABBIT.**—ANAPHYLACTIC SHOCK B8-415, B8-416, B8-4431, B8-4451.

**RAT.**—ANAPHYLACTIC SHOCK B8-4431, B8-4451.

**RED BLOOD CORPUSCLES.**—V. ERYTHROCYTES.

**RICIN.**—ANTITOXIN B8-9711.

**ROEMER.**—STANDARDIZATION ANTITOXIN B8-914.

**ROUX.**—STANDARDIZATION ANTITOXIN B8-911.

**SACHS AND GEORGI TEST.**—SYPHILITIC SERUM B10-39.

**SAUSAGE MEAT.**—ANTIGEN B9-762—COMPLEMENT FIXATION B9-761—PRECIPITIN TEST B10-8431.

**SCHICK.**—TEST B8-52, B8-915.

**V. SEPTIQUE.**—ANTITOXIN B8-95.

**SERUM.**—REACTIONS V. BLOOD SERUM.

**SNAKE VENOM.**—ANTIVENIN B9-2—MINIMUM LETHAL DOSE B9-222.

**SPOROTHRIX.**—AGGLUTINATION B8-28—ANTIGEN B8-28.

**SPUTUM.**—ANTIGEN B10-721 to B10-723.

**STAIN.**—ALKALINE METHYLENE BLUE B10-6421—CARBOL THIONIN B10-6416.

**STALAGMOMETER.**—SERUM TEST B10-5.

**STANDARD.**—AGGLUTININ UNIT B8-1335—LOOP B9-4121.

**STANDARDIZATION.**—ABRIN ANTI-TOXIN B8-97—ANTIENDOTOXIN B8-7  
—ANTIGEN B9-322, B9-716 B10-52—ANTIGEN  
AGGLUTINABILITY B8-133—ANTIGEN OPACITY  
B8-132—ANTIGEN WASSERMANN B9-716, B10-924,  
B10-925—ANTIHÆMOLYSIS B8-8122—ANTI-SERA  
BLOOD STAIN B9-751—ANTI-TRYPSIN B9-1—  
ANTIVENIN B9-2—BACTERIOLYTIC SERUM  
B9-421—BOTULINUS ANTITOXIN B8-93—  
COLONY COUNT B9-322—COMPLEMENT B9-715,  
B10-916—CROTIN ANTITOXIN B8-9711—DIPH-  
THERIA ANTITOXIN B8-91—HÆMOLYTIC  
SERUM B9-713, B10-41, B10-915—HAY FEVER  
ANTITOXIN B8-532, B8-98.—B. CEDEMATIENS  
ANTITOXIN B8-96—PRECIPITIN SERUM B10-823  
—RICIN ANTITOXIN B8-9711—V. SEPTIQUE  
ANTITOXIN B8-95—TETANUS ANTITOXIN  
B8-92—TRYPSIN B9-115, B9-122, B9-132—B. WELCHII  
ANTITOXIN B8-94.

**STAPHYLOCOCCUS.**—ANTIGEN B10-612—OPSONIC ACTION B10-612.

**STREPTOCOCCUS.**—AGGLUTINATION B8-19—ANTIGEN B8-19—COMPLEMENT  
FIXATION B9-7311.

**SUBCUTANEOUS TEST.**—ANIMAL B8-476—GLANDERS B8-481—KÖCH B8-475  
MALEIN B8-481—TUBERCULOSIS B8-475.

**SUPPURATION.**—DIAGNOSIS B9-1161.

**SYPHILIS.**—ANTIGEN MEIOSTAGMIN TEST B10-515—BROWN AND  
IYENGAR TEST B9-522—BRÜCK TESTS B10-321 to B10-322  
—BUTYRIC ACID TEST B10-371—COBRA VENOM REACTION  
B9-52—COMPLEMENT FIXATION B10-9—CUTANEOUS TEST  
B8-541—EPIPHANIN REACTION B10-21—FLOCCULATION  
REACTION B10-3—HERMANN AND PERUTZ TEST B10-33—  
HIRSCHFELD AND KLINGER TEST B10-34—KLAUSNER  
TEST B10-35—MEINICKE TEST B10-36—NOGUCHI FLOC-  
CULATION TEST B10-37—PORGES AND MEIER TEST B10-382  
—PORGES TEST B10-381—SACHS AND GEORGI TEST B10-39  
—WASSERMANN TEST B10-9—WEIL TEST B9-521.

**B. TETANI.**—ANTITOXIN B8-92—ANTITOXIN UNIT B8-9214—PRECIPITIN  
STANDARDIZATION B10-78—TOXIN STANDARD B8-9212

**THERMOPRECIPITIN TEST.**—B. ANTHRACIS B10-750—ANTHRAX ANTIGEN  
B10-752, B10-753

**THIONIN.**—CARBOL STAIN B10-6416

**THREAD.**—REACTION B8-153.

**TITRATION.**—SERA v. STANDARDIZATION.

**TITRE.**—V. ALSO STANDARDIZATION UNIT—BACTERIOLYTIC SERUM B9-4211—HÆMOLYTIC SERUM B9-7131, B9-7133.

**TOXIN.**—V. ANTITOXIC ACTION—STANDARD TETANUS B8-9212.

**TRANSFUSION.**—TEST B8-33, B10-42.

**TRYPSIN.**—SOLUTION B9-111—STANDARDIZATION B9-115, B9-122, B9-132.

**TUBERCULIN.**—CONJUNCTIVAL TEST B8-4601, B8-477—CUTANEOUS TEST B8-471—FOR CONJUNCTIVAL TEST B8-4774—INTRACUTANEOUS TEST B8-473—INTRACUTANEOUS ANIMAL TEST B8-474—NASAL TEST B8-478—PERCUTANEOUS TEST B8-472—SUBCUTANEOUS TEST B8-475—SUBCUTANEOUS ANIMAL TEST B8-476—TEST B8-47—URETHRAL TEST B8-479—VAGINAL TEST B8-479.

**B. TUBERCULOSIS.**—AGGLUTINATION B8-1211, B8-22—ANTIGEN B8-22, B9-738, B10-614—COMPLEMENT FIXATION B9-738—OPSONIC ACTION B10-614, B10-642—PRECIPITIN TEST B10-74—STAINING B10-642.

**TYPE.**—MENINGOCOCCUS B8-1832, B8-3201—PNEUMOCOCCUS B8-21, B10-72—SEROLOGICAL B8-3201.

**TYPHOID FEVER.**—CONJUNCTIVAL TEST B8-491—CUTANEOUS TEST B8-492—ORGANISMS AGGLUTINATION B8-1128.

**B. TYPHOSUS.**—ANTIENDOTOXIN B8-73—COMPLEMENT FIXATION B9-7311.  
**UNIT.**—AGGLUTINATION B8-1124, B8-1335, B8-1344—ANTICOMPLEMENTARY B10-924—ANTIENDOTOXIN B8-725, B8-711—ANTIGEN B9-7167—ANTITRYPSIN B9-123, B9-132—ANTIVENIN B9-2211, B9-2221—BACTERIOLYTIC SERUM B9-4211—DIPHTHERIA ANTITOXIN B8-9135—HÆMOLYTIC COMPLEMENT B9-62, B9-7154—HÆMOLYTIC SERUM B9-7131, B9-7133, B10-4113, B10-4122—POLLEN ANTIGEN B8-5312—TETANUS ANTITOXIN B8-9214—TRYPSIN B9-115, B9-122, B9-1421—VENOM B9-222.

**URETHRAL TEST.**—TUBERCULOSIS B8-479.

**VAGINAL TEST.**—TUBERCULOSIS B8-479.

**VENOM.**—ANTIVENOMOUS ACTION B9-2.

**VIBRION SEPTIQUE.**—V. SEPTIQUE.

**WASSERMANN.**—REACTION B9-8, B10-9—TEST B10-917, B10-918, B10-926, B10-927—TEST ANTIGEN B10-917, B10-921 TO B10-923—TEST EXTRACT B10-917, B10-921 TO B10-923—TEST FLEMING B10-95—TEST HECHT B10-94—TEST NOGUCHI B10-93.

**WEIL.**—TEST B9-521.

**B. WELCHII.**—ANTITOXIN B8-94.

**WRIGHT, A. E.**—AGGLUTINATION TEST B8-12—ANTITRYPTIC ACTION B9-14—BACTERICIDAL ACTION B9-32.

**WRIGHT AND DOUGLAS.**—OPSONIC ACTION B10-6.

# ON THE BEHAVIOUR OF PARAMECIUM CAUDATUM TOWARDS THE CINCHONA ALKALOIDS

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[ Received for publication, June 28, 1921. ]

FOR a long time, it had been known that paramecium varied considerably in their behaviour towards poisonous drugs. Jennings 1906 showed that the variations were due to the amount of  $\text{CO}_2$  present in the culture media, and considered that these ciliates should only be tested, after the culture was freed from carbon dioxide by blowing air through the media. Dixon 1920 suggested that these differences in resistance could be overcome to a certain extent by putting up a quinine control as a comparison with the drug to be tested. Dale commenced investigating the cause of this variability but had to abandon the work owing to the war. Before I started testing the alkaloids of cinchona bark on these protozoa Dr. Dale very kindly gave me his notes, and throughout the work, helped me with advice and suggestions. It is usually stated that paramecium can be grown from tap water or hay infusions. Dobell informs me that this is not the case, paramecium are found in stagnant water, near dung heaps, etc., and consequently a great deal of the work stated to have been done on this ciliate, has been done on Colpoda, Bodo, etc., these protozoa are more commonly found in tap water and hay infusions. In a former paper on snake venoms, Acton and Knowles 1914 were able to construct a dose death-time curve by injecting rats with known doses of dried cobra venom and recording the time to death. We were able by means of this curve to estimate the amount of venom injected by a cobra at an experimental bite. I had hoped to

construct a similar type of curve for paramecium, arrive at the factors concerned with these variations, and make a more accurate test.

*Technique.*—The ciliate used throughout this work was *Paramecium caudatum* isolated for me by Clifford Dobell, Esq., F.R.S., from a single individual. I am indebted to him for a great deal of help during the course of the work. This race of paramecium had been observed by Dobell for over three years and he has never seen conjugating forms. (See also Woodruff 1912.) The rate of division of these protozoa is once every 20 hours (provided temperature and food conditions are favourable). The culture medium is made by infusing a small handful of chopped hay in  $\frac{3}{4}$  of a litre of tap water. The hay infusion is boiled for 3 minutes in order to destroy all protozoa and non-sporing bacteria; prolonged boiling kills the sporing bacteria, and the paramecium from want of food grow very slowly in these sterile cultures. The hay infusion is then allowed to cool to room temperature, and inoculated with 5 c.c. of stock culture; a thick culture was obtained in 8-10 days (by using a massive insemination). The paramecium grow better in deep cultures with the surface well exposed to air. The pigment of the infusion acts as a fairly good indicator, the dark brown cultures are sensitive, and the lighter cultures more resistant. Peters 1920 claims to cultivate these ciliates free from bacteria in a medium containing amino-acids. At the beginning of this research, small volumes of culture and alkaloid solutions were examined on a well slide, but this technique was abandoned because (under drop conditions) it was very difficult to get complete mixing of the two fluids. The error was overcome by using larger volumes, *i.e.*, 1 c.c. of culture and 1 c.c. of alkaloid solution. A binocular dissecting microscope with No. 1 oculars and low objectives was used for examining the paramecium, this gave a large field and sufficiently high magnification and it was possible to keep a volume of 2 c.c. under observation. One c.c. of the test alkaloid solution was placed first in the small 2 c.c. glass cell and 1 c.c. of culture added, and well stirred for a few seconds. This technique was altered when investigating the factors concerned in the construction of the dose-death time curve, *e.g.*—

*The absorption time* was found by filling the small 2 c.c. glass cell with the test alkaloid solution, 10 cmms. of paramecium culture were taken up into a pipette and an air space left to separate the culture from the alkaloid solution. The end of the pipette was inserted into the alkaloid solution and moved about until it was under the centre of

the microscope field. This small volume, containing about 8-10 parameciums, was blown into the alkaloid solution and one could observe how long it took to kill the ciliates.

*The lethal concentration.*—In a series of twelve or more test tubes 1 c.c. of the different concentrations of alkaloid was placed in each. The test concentrations ranged from 1-1000 to 1-500000 of anhydrous base. The paramecium culture was taken up in a 10 c.c. pipette from  $\frac{1}{2}$ " below the surface, where the ciliates were most numerous, and 1 c.c. of culture added to each test tube making the final concentrations range from 1-2000 to 1-1000000. The end of the pipette should be as near as possible to the surface of the alkaloid solution, otherwise two sources of error occur, the  $P_H$  may be altered if the culture is aerated by dropping from a height, or drops may adhere to the sides containing paramecium not in contact with the alkaloid solution. Twenty to 24 hours later, the tubes should be well shaken as the paramecium sometimes remain motionless. The contents are then poured into a 2 c.c. glass cell and examined under the microscope. After each examination the glass cell should be washed and dried, otherwise paramecium may be carried over. By using 2 c.c. of fluid in ordinary sized test tubes not much  $CO_2$  is lost.

*The death interval* was found by the time it took to kill every individual in 1 c.c. of a thick culture when added to 1 c.c. of the particular concentration of the alkaloid. The live cell was constructed as follows:—four pieces of glass tubing  $\frac{5}{8}$ " wide and  $\frac{3}{4}$ " high each of over 2 c.c. capacity were cut, and the edges ground flat. The four cells were cemented together on the centre of a piece of  $\frac{1}{8}$ " plate glass 6" long and 2" broad, with Canada Balsam so that the microscopic field included a portion of each cell; the four could be kept under observation (at the same time). Four simultaneous observations could thus be carried out on a particular dilution or on four different dilutions. With a little experience, one could choose the strength of concentrations or space the time-intervals, so that a sufficient interval of time was given to thoroughly examine the cell and see that every individual was dead.

*The hydrogen ion concentration ( $P_H$ ) of the culture* should be taken as it gives the range of the test concentrations and an approximate idea of the death time. The method used for testing the  $P_H$  was one devised by Dale and Lovatt-Evans 1920. The collodion dialyser was filled to the top with the culture (about 3 c.c.), then corked, and allowed to dialyse for five minutes into 1 c.c. of freshly boiled distilled water. The

dialyser and its contents were then removed, '08 c.c. of a '02% solution of neutral red in distilled water was next added to the dialysate and immediately covered with a layer of liquid paraffin to prevent loss of CO<sub>2</sub>. The colour produced by the Neutral Red in the dialysate was matched with the tint in a standard phosphate solution.

"THE CAUSE OF VARIATIONS IN THE RESISTANCE OF PARAMECIUM."

(I) *Race*.—Dale and Dobell 1916 in a study on the therapeutic action of emetine in amœbic dysentery found that the different strains of *entamoeba histolytica* varied very considerably in their powers of resistance towards this alkaloid. This factor did not apply in my experiments as the ciliates used were all derived from a single individual.

(II) *Individual resistance*.—I noticed, when recording the time to death, that a few individuals took twice or three times as long to die as the rest. For example, at a concentration of 1-10000 quinine, the majority, some two to three hundred individuals, died in three minutes, five or six survived for six minutes, and one or two individuals struggled on for ten minutes. The average sized paramecium seemed to die first, and the survivors were nearly always the extreme individuals. Again, in the test for the minimum lethal concentration, when the series of test tubes were examined next day, a concentration was found at which every individual was killed (certain lethal concentration); below this concentration a certain percentage of the individuals survived (sub-lethal concentrations). Finally in still lower concentrations every individual was alive after 20-24 hrs. (non-lethal concentrations).

The following example of a test may be given as an illustration :—

Final conc. of	1-20000	anhydrous quinine base.	No survivors	} Certain lethal concentration.
" "	1-30000	" "	" "	
" "	1-40000	" "	12.5% survived	} Sub-lethal concentration.
" "	1-50000	" "	16.6% "	
" "	1-70000	" "	50% "	
" "	1-100000	" "	75% "	
" "	1-200000	" "	all alive	} Non-lethal concentration.
" "	1-250000	" "	" "	

We therefore see that there are certain individuals who take a longer time to die in lethal concentrations and are also able to resist sub-lethal concentrations.

(III) *Light*.—The difference in action of acridine on paramecium when tested in the light and dark is quoted to show that light

plays a part in explaining the variations in sensitiveness of these protozoa. The four cinchona alkaloids were tested in the light and dark and this difference in lethality was not detected. The sulphates of cinchonine and cinchonidine are not fluorescent whilst the sulphates of the other two are fluorescent. Again the actinic power of the light was tested on several occasions when these tests were being carried out and no obvious association was seen.

	Actinic value	CONC. ANHYDROUS QUININE BASE				Time to death in minutes.
		1-10000.	1-12000	1-20000	1-24000	
Original culture ..	20	10½ Mins.	14½ Mins.	26 Mins.	66½ Mins.	
Ditto ..	15	12 ..	16½ ..	29½ ..	52 ..	
Same culture exposed 2 hours in a Petri dish ..	12	1½ ..	1½ (undr.)	3 ..	3½ ..	
Same culture after 3 hours exposure ..	13	1½ ..	1½ ..	3½ ..	3 ..	

(IV) *The hydrogen ion concentration ( $P_H$ ) of the culture media.* When 50 c.c. of paramecium culture were placed in a flat Petri dish, this was done in order to avoid contaminating the stock culture. I noticed with the same concentration of alkaloid, the longer the culture was exposed to air the shorter became the interval to death. The following experiment shows one of these experiences:—

50 c.c.s. of a stock culture from a litre flask were placed in a Petri dish.—6 in. diameter. At intervals of half an hour, 1 c.c. of the exposed culture was added to 1 c.c. of the following concentrations of quinidine, and the time to death of the last individual noted.

TABLE I.

	FINAL CONCENTRATION OF QUINIDINE ANHYDROUS BASE.				Time to death in minutes.
	1-10000	1-12000	1-20000	1-24000	
Original culture in flask ..	Mins. 12	Mins. 22½	Mins. 30	Mins. 48	
After ½ hour's exposure ..	12½	16½	22	42	
.. 1 hour ..	7	15	19	27	
.. 1½ ..	7½	13	17	26½	
.. 2 ..	4	7	16	19	
.. 2½ ..	4½	4	6½	11	
.. 3 ..	3½	4	6	9	
.. 3½ ..	2	2½	3½	7½	

This experiment was repeated on several occasions and similar results were obtained, showing that the increased sensitiveness of the paramecium was associated with exposure of thin layers of culture to a large surface of air. Jennings 1906 observed that the resistance of paramecium was largely conditioned by the amount of  $\text{CO}_2$  produced in the media. The following experiment was performed to show this effect:

*Experiment II.*—One c.c. of a 1-10000 quinidine was mixed with 1 c.c. of the following cultures giving a concentration of 1-20000 of the anhydrous base in each c.c. of the mixture. Three different lots of 50 c.c. from the original stock culture were treated as follows:—

- (I) 50 c.c. were placed in an open dish and exposed to air.
- (II) 50 c.c. were placed in a dish and had  $\text{CO}_2$  blown over it for 40 seconds, and then covered with a glass lid. Each time the culture was tested,  $\text{CO}_2$  was again added for 40 seconds.
- (III) 50 c.c. were placed in a small Ehrlenmayer's flask of 50 c.c. capacity with a narrow neck and covered with a watchglass.

TABLE II.

The results were as follows:—

Time of test.				(I) Open Petri dish.	(II) $\text{CO}_2$ dish.	(III) Ehrlen- mayer's flask.	Original culture.	Time to death in minutes.
Hrs. Mins.				Mins.	Mins.	Mins.	Mins.	
After	0	50	..	7½	366	16	70	
"	1	40	..	4	158	..	68	
"	2	45	..	2½	113	..	..	
"	3	15	..	1½	75	..	..	
"	4	0	..	1¼	Left open till next morning.	15	..	
"	24	0	..	1½	1½ mins.	12	65	
"	48	0	..	5	required with $\text{CO}_2$ 125 mins.	18	60	

At this stage I consulted Dr. Lovatt-Evans about the cause of this increased resistance of the paramecium after blowing  $\text{CO}_2$  over the cultures. He suggested that the cause may be due to the difference in the hydrogen ion concentration.

The next day Evans kindly estimated the hydrogen ion concentration ( $\text{P}_\text{H}$ ) of these cultures. The  $\text{P}_\text{H}$  of the exposed Petri dish was over

9.7, that in the 50 c.c. Ehrlenmayer's flask  $P_H$  7.76, and the original culture  $P_H$  7.32.

The following experiments clearly show that the variations in the resistance of different cultures is closely associated with the  $P_H$  of the media in which the paramecium are growing.

#### (A) ON VARIATIONS IN DEATH TIME

The death times were noted by testing 1 c.c. of culture with 1 c.c. of 1-10000 quinidine, this gave a concentration of 1-20000. Evans kindly estimated all the  $P_H$  of these cultures.

TABLE III.

Culture Source.	$P_H$ Concentration.	Time to death.
(a) Culture with CO <sub>2</sub> blown over ..	7.12	Survived 24 hours. 120 mins. 82 .. 4½ ..
(b) Ditto ..	7.27	
(c) Ditto ..	7.32	
(a) Original culture ..	7.52	
(a) After 16 hours exposure ..	8.08	

#### (B) ON VARIATIONS IN THE LETHAL CONCENTRATION.

The following cultures were tested, 1 c.c. of the various concentrations of quinine was mixed with 1 c.c. of culture and allowed 20 hours contact. They were then examined to see if all the individuals were dead. The results are shown in the following table:—

TABLE IV.

*Shows the minimum lethal concentration that is required to kill cultures of paramecium with different  $P_H$ .*

Source of culture.	$P_H$ Concentration	1 10000.	1 20000	1 25000.	1 27500.	1 30000	1 35000.	1 4000	1 50000.	1 70000	1 100000	1 200000.	Concentration of quinine in hydrolys.
CO <sub>2</sub> blown through culture A ..	7.12	+	75% ..	-	-	-	-	-	-	-	-	-	....
CO <sub>2</sub> blown through culture B ..	7.27	+	50% .. 66%	80%	-	-	-	-	-	-	-	-	....
Original culture (A)	7.52	+	+	+	+	+	25% .. 28% .. 75%	80%	-	-	-	-	....
O <sub>2</sub> blown through culture (B) .	7.66	+	+	+	+	-	-	16% .. 33%	-	-	-	-	....
Culture (B) ..	7.79	+	+	+	+	+	+	+	+	+	75%	-	....
Nitrogen blown through culture B	7.86	+	+	+	+	+	+	+	+	+	80%	-	....

The sign + = all dead, - = all alive, the % roughly estimates the number of survivors.

This experiment shows that the concentration of quinine that causes the death of every paramecium "Certain lethal concentration" varies with the hydrogen ion concentration of the cultures. When the  $P_H$  was 7.27, .1 millegramme of anhydrous quinine was required in each c.c. to kill every individual, whilst at a  $P_H$  of 7.86 as little as .015 millegrammes per c.c. killed with certainty. In other words, when the culture was acid the paramecium were capable of surviving six lethal doses. I must thank Dr. Lovatt-Evans for estimating the  $P_H$  of these cultures, and for his help when I was trying to account for the cause of these variation in death time and lethal dose.

"THE DOSE DEATH-TIME CURVE."

Madsen and Nogouchi, 1907, found in the guinea-pig, that a relationship existed between the dose and death time and could be plotted as an asymptotic curve. Acton and Knowles, 1914, obtained identical findings using rats, rabbits and monkeys in their venom experiments, and other workers have obtained similar results when injecting toxins into guinea-pigs and other laboratory animals. Thus confirming the asymptotic relationship that exists between dose and death time for mammals in general. I therefore assumed that a similar toxicity curve could be constructed for paramecium. This relationship we had previously represented by the equation

$$(x - a)(y - b) = K.$$

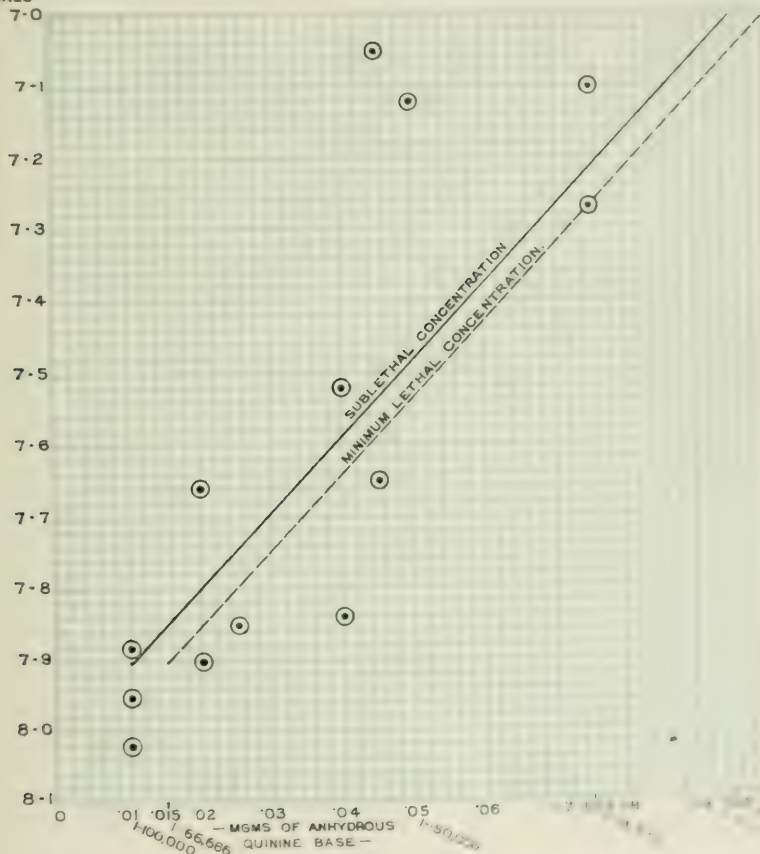
where  $x$  = the dose of alkaloid in mgrms. per c.c.  $y$  = the time to death in minutes. These two factors could readily be obtained by experiments.  $(a)$  = absorption time of the alkaloid and  $(b)$  the sub-minimal lethal concentration that just fails to kill every individual,  $K$  = a constant which is at present unknown. In order to eliminate the value of  $K$  we must first determine the values of  $a$  and  $b$ .

*Sub-minimal lethal concentration.*— $(a)$  I have already discussed the minimum lethal concentration, and showed that it varied with the  $P_H$  of the culture. Thus at a  $P_H$  of 7.12 it was 1-10000 and at a  $P_H$  of 7.86 it was 1-70000 or differences of .1 and .015 mgrms. of anhydrous base per c.c. In alkaline cultures, differences of .005 mgrms. of anhydrous quinine base can be detected, so if this difference is taken as the sub-lethal dose, the sub-lethal dose will vary from 0.1 to .095 mgrms. in the range of  $P_H$  from 7 to 7.9. Graph I shows these two lines, the lethal and sub-lethal dose, as running parallel to one another. The actual observations have been shown as a dot within a circle.

546

Graph I—Shows the relationship of the minimum and sublethal concentration of Quinine with the  $P_H$  of the Culture.

HYDROGEN  
ION  
CONCENTRATION  
 $P_H$   
OF CULTURES



The dotted line = Minimum lethal concentration. The solid line = The approximate sublethal concentration. The actual observations are shown thus (o)



We see that below  $P_H$  7.9 the sub-lethal dose is about .01 mgrms. Between  $P_H$  7.9 and 7.5 the observations lie fairly evenly about this line, between  $P_H$  7 and 7.3 the observations lie some distance from the line, and may be explained by the loss of  $CO_2$  that occurs when such cultures are exposed to air in test tubes. By the aid of the graph it is possible to make the necessary corrections in the sub-lethal dose with a given  $P_H$ .

*The absorption time.*—(b) The exact time was estimated by blowing 20 cmm. of paramecium culture in 2 c.c. of alkaloid solution, and counting the number of seconds it took to kill. The following is a result obtained on a stock culture:—

Concentration					Death in seconds
1-100 anhydrous quinine base	..	..	..	..	Instantaneously
1-200	..	..	..	..	1 or 2 seconds
1-300	..	..	..	..	3 seconds
1-400	..	..	..	..	5 ..
1-500	..	..	..	..	8 ..

The following cultures were then tested, to see the effect of the  $P_H$  on the different cultures:—

Cultures.		CONCENTRATION OF ANHYDROUS QUININE BASE		
		1-200	1-400	1-800
CO <sub>2</sub> .	Culture I	$P_H$ over 6.5		
	Culture I	$P_H$ 7.23		
	Culture II	$P_H$ 7.76		
	Culture III	$P_H$ 8.08		
		inst.	5 sec.	40 sec.
		inst.	inst.	10 sec.
		inst.	inst.	inst.
		inst.	inst.	inst.

A culture exposed for over 24 hours to the air in a flat Petri dish,  $P_H$  over 9.5, death occurred instantaneously in a dilution of 1-80000 of quinidine. Therefore in strong concentrations, the absorption time is instantaneous, and in alkaline cultures this degree of lethality is maintained in much weaker dilutions. Unless small volumes of culture are blown into a large volume of alkaloid solution it is impossible to accurately time the deaths, as they occur so quickly. With the rest of my experiments I used 1 c.c. of culture and 1 c.c. of alkaloid solution and anything under six seconds, *i.e.*, 1/10 of a minute, was considered as instantaneous for the time was occupied in mixing and placing the preparation under the microscope.

Now what does this rapid rate of absorption mean? We know that quinine in an alkaline solution is transformed into the base which

is an alkali behaving like ammonia. It is well known that ammonia has the power of rapidly penetrating living cell membranes.

We now show by these experiments that quinine base can penetrate the cells just as rapidly as ammonia. Quinine has two tertiary N. atoms and may possibly behave like  $\text{NH}_3$ . In acid cultures strong solutions are required to kill instantaneously. Quinine base does not dialyse through a collodion membrane, whilst the acid salts do, and later on we will see that this fact has an important bearing on the penetration of the base through the erythrocyte stroma.

*The Evaluation of K:—*

As we know the value of  $a$  and  $b$  in this equation we can obtain the value of  $K$  easily by taking four points on the curve at a particular  $P_H$  and then find the value of  $K$ . This has been done in the following:—

TABLE V.

*Shows the calculated and observed values of the close death time of the different cultures with  $P_H$  varying from 6.5 to 9.5 and over.*

	PH OVER 9.5		PH CULTURE 8.08		PH	7.23	PH	OVER 6.5
	Observed time.	Calculated K°.04	Observed time in mins.	Calculated K=2.3	Observed in mins.	Calculated K=2.5	Observed in mins.	Calculated K=7.8
1-2000 ..	Inst.	.18 sec.	5 5½ 6	4.8 mins.	11 10 8½	6 mins.	16 15 13	19.3
1-4000 ..	Inst.	.27 "	9 8 8½	9.8 "	17 16½ 13	15	40	50.0
1-6000 ..	Inst.	.38 "	10 12½ 11½	15.3 "	21 22 18	27.5	105	109.5
1-8000 ..	10 sec.	.44 "	16 13 15	21 mins	30 28 30	50.1	—	260
1-10000 ..	25 "	.5 "	—	27 "	—	92.6	—	560
1-12000 ..	35 "	.68 "	—	33 "	—	250	—	∞
1-14000 ..	35 "	.8 "	—	37.8 "	—	∞	—	—
1-16000 ..	65 "	.9 "	—	49 "	—	—	—	—
1-18000 ..	95 "	1.1 mins.	—	57 "	—	—	—	—
1-20000 ..	2½ mins.	1.2 "	—	65.5 "	—	—	—	—
1-40000 ..	5½ "	4.1 "	—	230.1 "	—	—	—	—
1-50000 ..	—	9	—	460.5 "	—	—	—	—
1-80000 ..	live after 75 mins.	∞	—	∞	—	—	—	—

The observed and calculated values fit fairly well, but there is a discrepancy in the longer death interval owing to the  $P_H$  of the mixture altering by the loss of  $\text{CO}_2$ . The important point this table shows is that the death interval is also correlated with the  $P_H$ . In alkaline cultures the instantaneous lethal action of quinine base is remarkable, in the acid solutions the action of quinine is much weaker and is possibly due to the formation of insoluble carbonates by the excess of  $\text{CO}_2$  present, thus interfering with the penetration of the alkaloid.

At the same dilution with different cultures owing to the extreme variability of the death time there is a rapid loss of  $\text{CO}_2$  when these mixtures are exposed to air. As a measure of toxicity, this test would require a number of observations and great care. For these reasons I abandoned the method and used instead the minimum lethal concentration. The other alkaloids cinchonine, quinidine, cinchonidine and the hydro-alkaloids behave like quinine, and similar curves could be constructed for them.

#### THE TOXICITY OF THE MAIN CINCHONA ALKALOIDS.

The  $P_H$  of the culture should first be estimated, we have already seen that the hydrogen ion concentrations of the cultures have a close association to the lethal dose of these alkaloids. Thus a culture with a  $P_H$  of 7 is very insensitive, whilst a culture of  $P_H$  8 is killed by a sixth of the lethal dose required in acid cultures. When the  $P_H$  is about 7, there is a good deal of  $\text{CO}_2$  present, and care should be taken that the 1 c.c. volume does not lose much  $\text{CO}_2$ . Again if the culture is exposed in shallow layers to a large air surface,  $\text{CO}_2$  is rapidly given up, this loss is to a large extent overcome by doing the tests in ordinary test tubes. The first test was done on a culture with a  $P_H$  of 7.25 and for each alkaloid twelve different concentrations were put up for the test. Only the minimum lethal concentration, that is sufficient to kill every individual in 1 c.c. is shown in the Table VI.

TABLE VI.

*Shows the relative toxicity of the different cinchona alkaloids when tested on a paramecium culture  $P_H$  7.25.*

Dextrorotatory, cinchona alkaloids (A) Cinchonidine series.	M. L. Dose.		Levorotatory cinchonines, (B) Cinchonidine series.	M. L. Dose.	
	Amount of anhydrous base.	Equivalent concentra- tion.		Amount of anhydrous base.	Equivalent concentra- tion.
Cinchonine ..	.3 mgm. per c.c.	1-3000	Cinchonidine ..	.1 mgm. per c.c.	1-10000
Hydro-cinchonine ..	.35 " "	1-2800	Hydro-cinchonidine ..	" "	1-10000
Quinidine ..	.1 " "	1-10000	Quinine ..	1 " "	1-10000
Hydro-quinidine ..	.15 " "	1-7000	Hydro-quinine ..	.15 " "	1-7000
Ethyl hydrocupre- idine ..	.025 " "	1-40000	Ethyl cupreine ..	.025 " "	1-25000

In this test cinchonine and hydro-cinchonine were found to be the least toxic for paramecium. Ethyl hydro-cupreidine was the most toxic, and ethyl hydro-cupreine (optechin) the next. Between quinidine, cinchonidine and quinine no differences could be seen. The hydro-alkaloids were all slightly weaker in their action.

The next test was done on a culture which was more alkaline and the results were as follows :—

TABLE VII

*Shows the relative toxicity of the different cinchona alkaloids when tested on a paramecium culture P<sub>H</sub> 7.79.*

Dextrorotatory Cinchona alkaloids. (A) Cinchonine series.	MIN. LETHAL DOSE.		Lævorotatory isomerides. (B) Cinchonidine series.	M. LETHAL DOSE.	
	Amount of anhydrous base.	Equivalent concentration.		Amount of anhydrous base.	Equivalent concentration.
Cinchonine ..	.25 mgms. per c.c.	1-4000	Cinchonidine ..	.02 mgms. per c.c.	1-50000
Hydro-cinchonine ..	.25 .. .. .	1-4000	Hydro-cinchonidine ..	.035 .. .. .	1-28000
Quinidine ..	.01 .. .. .	1-100000	Quinine ..	.020 .. .. .	1-50000
Hydro-quinidine ..	.015 .. .. .	1-65000	Hydro-quinine ..	.035 .. .. .	1-28000
Ethyl hydro-cupreidine ..	.0045 .. ..	1-220000	Ethyl hydro cupreine ..	.015 .. .. .	1-28000

We again see that when the culture is alkaline, these drugs kill in much weaker concentrations, but the order of relative toxicity is still maintained.

Thus cinchonine and hydro-cinchonine are the least toxic, and Ethyl hydro-cupreine and cupreidine the most toxic. In the acid cultures we saw very little difference between the others owing to the coarser gradation that occurred at these stronger concentrations. In the alkaline culture with finer gradations we see that quinidine is the most toxic of these three alkaloids, then cinchonidine, then quinine. The hydro-alkaloids are all slightly weaker in their action. That a difference

does exist between quinine and quinidine I tested these two alkaloids with the following results :—

*Lethal Concentrations.*

	1 in 100000	1 in 70000	1 in 50000	1 in 45000	1 in 35000	1 in 33000	1 in 24000	1 in 25000	1 in 20000	1 in 10000	No. of observations.
Quinine	..	—	2	—	1	2	1	1	1	3	4
Cinchonidine	..	—	—	1	—	—	2	—	1	—	
Quinidine	..	3	1	—	—	1	2	1	2	4	2

On fourteen different occasions when quinine and quinidine were tested together on the same culture, in seven quinidine appeared to be more toxic, in three no difference was seen, and in four quinine was more toxic. The cultures varied in  $P_H$  between 6.5 and 7.8. The certain lethal concentrations of quinine at these different  $P_H$  varied from 1-10000 to 1-70000 and quinidine from 1-10000 to 1-100000. So that quinidine is slightly more toxic than quinine for these ciliates.

*The concentration of quinine and quinidine necessary to prevent multiplication.*

Dr. H. H. Dale, F.R.S., suggested to me that it would be worth testing the degree of concentration that would stop or decrease the rate of multiplication; as this concentration should be considerably lower than that required to kill outright. The following experiment was done to test this point. To 30 c.c. volume of sterile hay culture the necessary amount of quinine and quinidine was added, so that each c.c. of the culture contained a definite amount of anhydrous base. Nineteen cultures ranging from concentrations of 1-10000 to 1-1000000 of anhydrous base were put up for each alkaloid.

The cultivation was done in crystallising capsules of 40 c.c. capacity and covered with a watch glass to prevent evaporation. A definite volume (20 cmm.) of stock culture of paramecium was used for each insemination. Three counts were made on this volume of culture and the actual number of paramecium found were 45, 49, 46 or an average of



'Culture' was the control. The second 'CO<sub>2</sub> and Air,' in these test tubes the culture and alkaloid were first allowed contact for 5 minutes and then CO<sub>2</sub> was blown into the tubes for 20 seconds. The third set 'CO<sub>2</sub> sealed' was treated as two but the test tubes were sealed up with wax. The tubes were read after 20 hours with the following result:—

	1-12000.	1-11000.	1-10000.	1-18000.	1-20000.	1-40000.	1-60000.	1-80000.	1-100000.	1-120000.	1-110000.	1-160000.	1-180000.	1-200000.	Concentration of anhydrous quinine base.
Culture	+	+	+	+	+	*	+	+	—	—	—	—	—	—	
CO <sub>2</sub> and air	—	+	—	—	—	—	—	—	—	—	—	—	—	—	
CO <sub>2</sub> sealed	—	+	+	+	+	—	—	—	+	+	+	+	+	+	

+ signifies dead, — = alive. Control 'sealed up' were all dead.

The cultures marked with \* contained live paramecium as well as a number killed by the alkaloid, a few of the living ones were taken out, well washed in a sterile medium and then inseminated in some fresh sterile hay infusion. The paramecium taken from the three air cultures, 1-40000, 1-60000 and 1-80000, all multiplied in the new medium. The paramecium taken from the first and third CO<sub>2</sub> air cultures at concentrations of 1-12000, 1-16000 survived and multiplied. A CO<sub>2</sub> atmosphere alone was toxic to paramecium.

(B) *The cultivation of paramecium from lethal concentrations.* A sensitive culture, which had been exposed to air for some time, was mixed with a 1-100000 quinine and this concentration was found to kill in 4 hrs. 35 mins.; \* 5 hrs. 15 mins.; 5 hrs.; \* 5 hrs. 30 mins., an average of 5 hrs. 5 mins. After two hours' contact, eight paramecium were taken from these cultures marked with an asterisk, and washed free of quinine. On the seventh day, multiplication had occurred in both cultures.

On the same culture 1-20000 quinine was found to kill in the following time — 40 mins. \* 8½ mins., 8½ mins. \* 8½ mins. After two minutes' contact, eight paramecium were taken out from the cultures marked with an asterisk, washed and planted in a new culture. The cultures were sterile a month after. I repeated the test several times and it was impossible for the paramecium to survive an immersion in these strong concentrations.

*These experiments indicate that—*

- (a) It is possible to cultivate paramecium from sub-lethal concentrations, if they are washed free of the alkaloid. Altering the  $P_H$  of the culture by the addition of acids, one can cultivate individuals from sub-lethal concentrations that would otherwise have been fatal.
- (b) It is impossible to cultivate paramecium that have passed through strong concentration (*i.e.*, above the range which is affected by the  $P_H$  of the culture).

#### CONCLUSIONS.

(I) The variations, in the dose and death time of this selected strain of paramecium, are due to alterations in the hydrogen ion concentration of the culture media.

(II) The hydrogen ion concentration of these cultures varied mainly with the presence or absence of  $CO_2$  in the media. The  $CO_2$  is produced by the paramecium or bacteria present. A deep culture with a small surface exposed to air becomes more and more acid, whilst a shallow culture with a large air surface becomes alkaline in a few hours.

(III) An individual resistance of the paramecium is also observed in these tests, but the error can be neglected if a large number of individuals are used.

(IV) When paramecium are put in strong concentrations 1-100 to 1-1000 the absorption time is instantaneous, indicating that quinine rapidly penetrates the cell membrane.

V) The toxic concentrations of these alkaloids have three definite ranges, *viz.* —

- (a) Certain lethal concentration, where the alkaloidal concentration is too toxic to be influenced by alterations in the reaction of the medium, *i.e.*, 1-10000 or stronger with quinine and 1-20000 or stronger with quinidine.
- (b) The minimum lethal concentration is the lowest concentration necessary to kill every individual and varies with the  $P_H$  of the culture. Thus at a  $P_H$  of 7 the M.L. concentration is 1-10000 for quinine and 1-20000 for quinidine, and at  $P_H$  of 8 the M.L. concentration is 1-70000 for quinine and 1-100000 for quinidine.
- (c) Sub-lethal concentrations are toxic concentrations, weaker than the minimum lethal concentration sufficient to retard

the rate of multiplication, but not toxic enough to kill all the paramecium. Thus at a  $P_H$  of 8, in concentrations of 1-200000 and 1-250000 with quinine and quinidine respectively, multiplication was slowed; below these limits, the alkaloid has no effect on the paramecium.

(VI) The dose death time test is a bad one to employ for these protozoa owing to the great variations caused by the  $CO_2$  content of these cultures.

(VII) The dextrorotatory cinchona alkaloids, with the exception of cinchonine, are more toxic to these ciliates than their levorotatory isomerides. The order of toxicity is as follows. Ethyl hydro-cupreidine, Ethyl hydro-cupreine (optochin), quinidine, cinchonidine, quinine, and cinchonine. The corresponding hydro-alkaloids are slightly less toxic to paramecium than the natural alkaloid.

(VIII) Paramecium can always be cultivated from sub-lethal concentrations; but in lethal concentrations their survival depends largely on the  $P_H$  of the culture. The passage of paramecium through certain lethal concentrations is always fatal owing to the rapid absorption time even though they be removed and washed free of the alkaloid.

#### THE APPLICATION OF THESE RESULTS TO THE TREATMENT OF MALARIAL FEVERS.

On whatever forms of animal life a toxic drug is tested, the toxicity depends largely on the size of the dose given, and the method of administration. The actual lethal dose varies with the species and size of the animal. Clinically we know that in malarial fevers, a single dose however large (30 to 90 grains) is not sufficient to permanently cure. Therefore the concentration of alkaloid circulating in the blood must be under "the certain lethal limit" otherwise a large dose should kill all the parasites. The concentration of quinine found in my blood by King, 1920, after taking a single dose of 20 grains of sulphate was 1-150000; after 10 grains 1-250000; after 5 grains 1-378000, this represented about 3% of the quinine ingested. We know that small doses 1-3 grains are insufficient to control fever, as the concentration attained in the blood 1-500000 or less is probably non-toxic to the parasites. A cure in malarial fever only occurs after the treatment has been continued for some time (3 to 6 weeks), *i.e.* if the quinine concentration in the blood has been maintained between these limits of 1-150000 and 1-250000. We

know that when patients are placed on a course of 30 grains of quinine, the parasites rapidly disappear from the peripheral blood. Acton, 1920, calculated the rate of destruction for each cycle of the benign tertian parasite to be over 90% of the parasitic population, so quinine is probably acting on the malaria parasite in sub-lethal concentrations. With paramecium, if the alkaloid is allowed to act on the ciliate for several days, death occurs at a lower concentration, *i.e.*, 1-100000, whilst multiplication is slowed in dilutions of 1-250000. Quinine circulates in the blood as a base, *i.e.*, an alkali, and as such cannot dialyse through a collo-dion membrane. For this reason it probably does not penetrate the erythrocyte membrane. There is clinical evidence in support of this view, for if quinine is given some time after the rigor, the parasites are seen in the red blood cells and the next attack is not prevented, indicating that the interior of the erythrocyte is quinine free. The malarial parasite is a haemosporidian and lives in the red blood cell, only leaving it when the corpuscle ruptures, to cross the plasma and attach itself to another cell. We know that quinine acts most intensively on the merozoites, less on the trophozoites, and least on the schizonts and gametocytes. King and myself found the distribution of quinine to be the same in the plasma as in the corpuscles. This evidence seems to point to the fact that quinine in certain concentrations destroys the merozoites either as they enter the plasma or whilst attached to the erythrocytes. There are therefore two main physical conditions concerned in the destruction of the malarial parasite by quinine or other cinchona alkaloids, *viz.*,

(A) CONCENTRATION.

(B) ACCESSIBILITY.

With paramecium, we saw that in sub-lethal concentrations the protozoa could be successfully cultivated if taken out and washed free of the quinine; in lethal concentrations the successful cultivation depended on the  $P_H$  of the cultures. In lethal concentrations above the  $P_H$  range the paramecium invariably died. In 1920 I pointed out that multiplication of the benign tertian parasite must occur in spite of treatment. For if the concentration of quinine present in the plasma or on the corpuscles was not sufficient to kill all the merozoites, they could enter a quinine-free area, *i.e.*, the interior of the erythrocyte, and there continue multiplying until the next generation.

I found that the  $P_H$  of the paramecium cultures made a great deal of difference in the concentration required to kill these ciliates. In a culture of  $P_H$  7 the concentration required to kill was a 1-10000 quinine

but a 1-70000 dilution killed in a culture of  $P_{H}$  S. Lovatt-Evans estimated the  $P_{H}$  of the portal and carotid blood of a cat for me, he found the portal blood to be  $P_{H}$  7.22 and the carotid blood  $P_{H}$  7.35. After 55 minutes of anaesthesia the blood was more venous in colour, the iliac vein blood was now  $P_{H}$  7.12 and the carotid blood 7.14. During the absorption of food from the intestines, one would expect the portal blood to be more acid than the systemic blood. During the absorption of quinine from the intestines, the concentration of quinine base would be greatest in these veins. We know that the malarial parasites differ from each other as regards their site of multiplication. The malignant tertian parasites multiply in the deeper vessels, *i.e.*, mesenteric; and in heavy infections, sporulation may occur in other sites, *e.g.*, brain capillaries. Schizonts are rarely seen in the peripheral blood. The benign tertian parasite multiplies anywhere in the blood stream, and schizonts are usually found in the peripheral blood before the attack of fever, whilst in quartan infections schizonts are numerous and invariably found in the peripheral blood. The sterilizing value of quinine in these three infections varies in the following order of efficiency, 90% or over of cures in malignant tertian infections, 25% in benign tertian infections, and 15 to 20% in quartans. The higher concentration of quinine in the portal vessels acting on the site, where merozoites are set free, may possibly be the factor concerned with the enhanced rate of cures in malignant tertian infections. If this factor was the only one, we would expect in benign, tertian and quartan infections that the intravenous and intermuscular methods of giving quinine to be more efficacious than the oral method, but there is no difference in the way that quinine is administered. Quinine in malignant tertian infections acts directly on the site of multiplication in as strong a concentration as is possible in the blood stream, but in a more acid blood. Whether the concentration of the alkaloid or the  $P_{H}$  of the blood plays the major share in bringing about the remarkable sterilizing effect of quinine in this infection remains to be still proved. What paramacium do in acid or alkaline cultures is no indication what the malarial parasite will do under similar conditions. The solution lies in making observations on the  $P_{H}$  and quinine content of the blood of patients undergoing treatment; as well as estimating the variations in the  $P_{H}$  of the portal blood in animals during the course of digestion. There may be something in the old established practice of giving alkalies or magnesium sulphate with quinine, thereby reducing the acidity of the portal blood, and



# INFECTION WITH *NUTTALLIA NINENSE* AMONG HEDGEHOGS IN THE NORTH-WEST FRONTIER PROVINCE, INDIA.

BY

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[Received for publication, March 11, 1921.]

On two occasions (August, 1914 and August, 1915) I had the opportunity of examining smears from the blood and organs of two hedgehogs (*Erinaceus* sp.) at Kohat, North-West Frontier Province, and on both occasions, the animals were found to be infected with a species of piroplasm.

The parasite was almost certainly that described by Yakimoff<sup>(1)</sup> in the hedgehog (*Erinaceus europaeus*) from Saratow in Russia and called by him *Piroplasma ninense*, Yakimoff, 1909. A similar parasite was described by Galli-Valerio<sup>(2)</sup> in blood smears from a hedgehog (*Erinaceus algirus*) from Tunis, which he thinks is probably the same as the species described by Yakimoff, but if it turns out to be a different species, he wishes it to be called *Piroplasma weissii*.

In neither of the animals from Kohat was the infection severe. The parasites were found in smears from the blood, liver, spleen and bonemarrow and were most numerous in the spleen smears.

The blood smears showed a well-marked polychromasia, but there was no very evident poikilocytosis although the red blood cells showed considerable variations in size. (Figs. 27 and 30, Plate XXVII.)

Normoblasts were present in the blood in fair numbers and 'Howell-Jolly bodies' were present in spleen smears.

#### THE PARASITES.

The parasites occurred in the red blood cells. Yakimoff described parasites in erythroblasts, but none were found in this situation in my cases, possibly because the infections were not severe.

Apart from the 'division rosettes,' usually only one parasite was found in a cell, but occasionally more were present. (Figs. 7, and 35-37, Plates XXVI and XXVII.)

Both Yakimoff and Galli-Valerio described parasites free in the blood, but only on one occasion was I able to find a definite free parasite.

When stained with Giemsa's stain, the protoplasm took on a blue colour and was more condensed and deeply stained at the periphery while there was usually a lighter area, often unstained, in the neighbourhood of the nucleus. The protoplasm was voluminous in comparison with the nucleus, especially in the larger forms.

In some of the parasites the protoplasm had an irregular outline, these irregularities were probably due to the amœboid activity of the parasites. (Figs. 33 and 34, Plate XXVII.)

The nucleus stained a deep carmine red and consisted of a solid mass of chromatin usually round in shape but becoming elongated in the dividing forms. The position of the nucleus was usually peripheral, but in a few rare cases it was central. (Figs. 35 and 37 Plate XXVII.)

The intracellular parasites may be divided into (1) small-sized forms, (2) intermediate-sized forms and (3) large-sized forms—

(1) Small-sized intracellular parasites :

These occur in three shapes, (a) ring-shaped, (b) oval and (c) elongated or rod-like forms.

(a) The ring forms were of the 'signet-ring' type and varied in size from  $0.7\mu$  to  $1\mu$  in diameter. (Figs. 1 and 2, Plate XXVI.)

They usually occurred singly in a corpuscle but may be accompanied by other forms. (Fig. 37, Plate XXVII.)

(b) The oval or oat-shaped forms measured about  $1\mu$  long by  $0.5\mu$  broad (Figs. 3 and 4, Plate XXVI). This was the common type in 'division rosettes.' (Figs. 27-30, Plate XXVII). In some cases one end was more pointed than the other, but the typical piriform

### EXPLANATION OF PLATE XXVI

The figures were drawn with an Abbé camera-lucida using a Leitz 1" apochromatic objective with a Zeiss No. 12 compensating ocular. Magnification about 2500 times.

All the figures were stained with Giemsa's stain except figs. 31-34 which were stained with Leishman's stain.

Figures 1, 2, 5, 10, 11, 13, 15, 18, 19, 22, 23 and 28 are from blood smears; figs. 3, 4, 14, and 29 are from bonemarrow smears; figs. 31 to 34 are from liver smears, and the remaining figures from spleen smears.

Figs. 1 and 2. Small ring forms.

„ 3 and 4. Small oval forms.

„ 5 and 6. Small rod-shaped forms.

Fig. 7. Two intermediate-sized ring forms in the same cell.

„ 8. Intermediate-sized ring form.

Figs. 9 to 11. Intermediate-sized oval forms.

„ 12 and 13. Intermediate-sized rod-shaped forms of which fig. 13 shows commencing division of the nucleus and protoplasm.

„ 14 and 15. Large ring forms.

„ 16 and 17. Large oval forms.

„ 18 and 19. Large rod-shaped forms.

# EXPLANATION OF PLATE XXVI

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Fig. 1 and 2. Small ring forms.

" 3 and 4. Small oval forms.

" 5 and 6. Small rod-shaped forms.

Fig. 7. Two intermediate-sized ring forms in the same cell.

" 8. Intermediate-sized ring form.

Fig. 9 to 11. Intermediate-sized oval forms.

" 12 and 13. Intermediate-sized rod-shaped forms of which

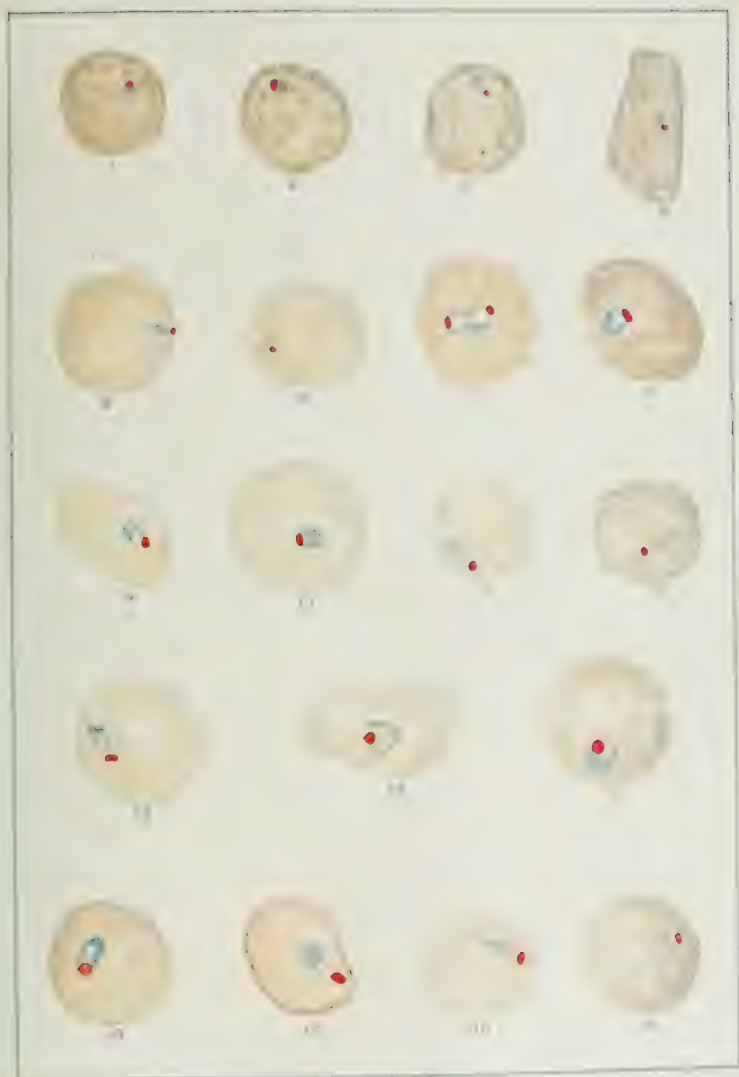
fig. 13 shows commencing division of the nucleus and proto-

plasm.

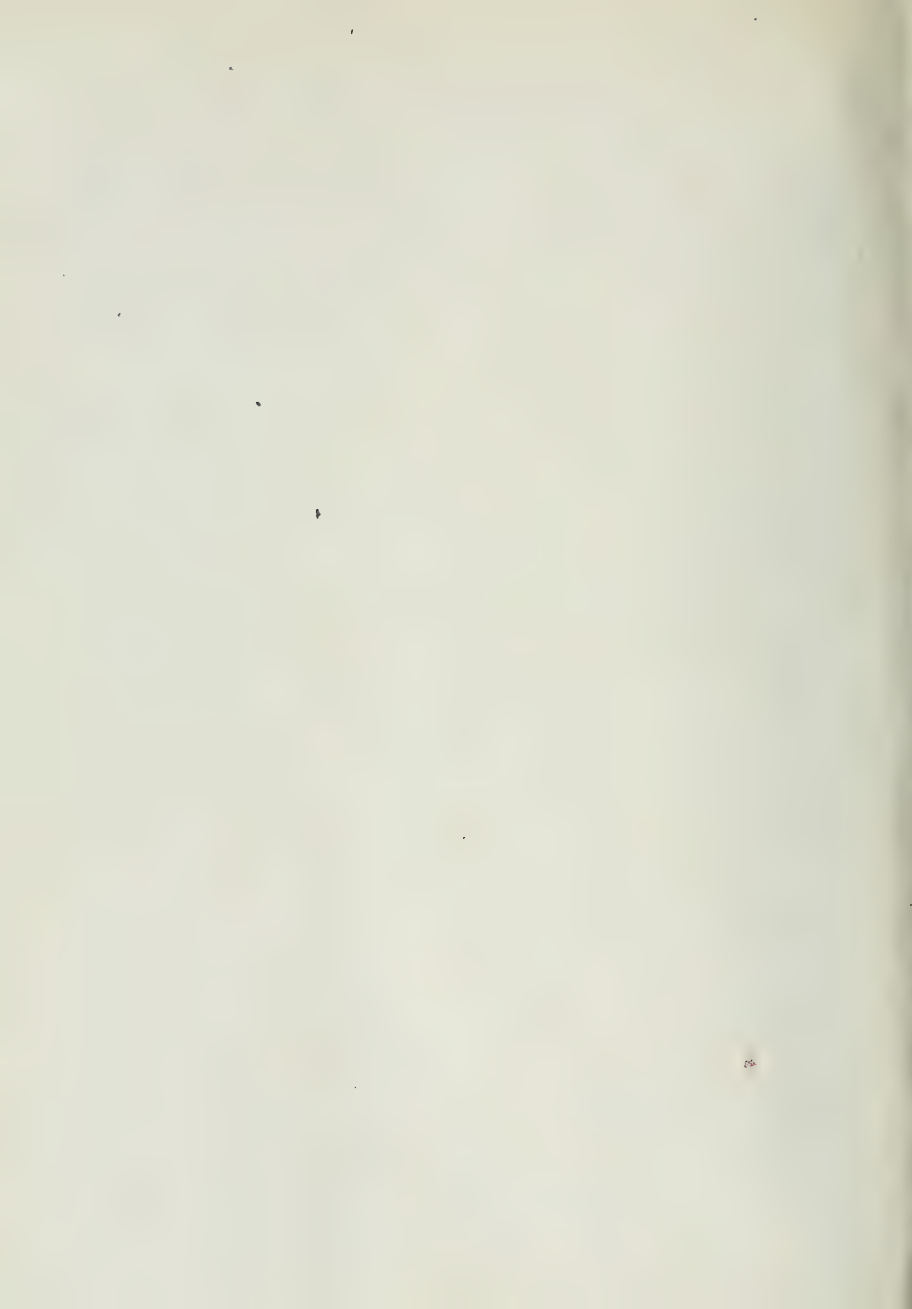
" 14 and 15. Large ring forms.

" 16 and 17. Large oval forms.

" 18 and 19. Large rod-shaped forms.



NUTTALLIA NINENSE



parasites with the nucleus at the broad end as seen in the genus *Piroplasma* (*Babesia*) were very rarely found.

(c) The elongated and rod-shaped forms resembled the oval forms but were more elongated. This type was rare. (Figs. 5 and 6, Plate XXVI.)

(2) Intermediate-sized intracellular parasites:

All gradations between the very small forms and the large forms were found. They also occurred in the same variety of shapes, *i.e.*, round, oval and rod-like. (Figs. 7—13, Plate XXVI.)

The oval forms tended to be rather piriform with the nucleus at the sharper end. Never more than two of this size of parasite were noted in a cell. (Figs. 7, 35 and 36, Plates XXVI and XXVII.)

(3) Large intracellular parasites:

These occurred in the same variety of shapes as the other two sizes of parasite.

(a) The ring forms were about  $2\mu$  in diameter. (Figs. 14 and 15, Plate XXVI.)

(b) The oval forms were intermediate in shape between the ring-shaped and the elongated forms. (Figs. 16 and 17, Plate XXVI.)

Some showed a more or less piriform outline with the nucleus at the sharp end. (Fig. 17, Plate XXVI.)

(c) The elongated forms corresponded to the 'cigar-shaped' forms of Yakimoff. Some of them measured  $3\mu$  long by  $1.5\mu$  broad. These forms were possibly due to the amœboid action of the parasite. (Figs. 18 and 19, Plate XXVI.)

These large forms seem larger than those described by Yakimoff and Galli-Valerio.

(4) Free forms of parasite:

Both Yakimoff and Galli-Valerio described free forms as occurring, but on only one occasion was a free form found in the specimens from Kohat. (Fig. 20, Plate XXVII.)

It is possible that the higher degree of infection made these forms commoner in their cases or that the more severe anaemia in their cases made the erythrocytes more fragile and so parasites were liberated from red cells burst in making the preparations.

Galli-Valerio figured an elongated free form which he states 'appears to represent a gamete' but in smears from the spleen and liver tissue of my cases I have seen bodies of a somewhat similar type which appeared to be blood platelets or portions of tissue cells.

## (5) Division forms :

Among both the intermediate and large-sized parasites, forms are found in which the nucleus clearly shows commencing division (Figs. 23 and 24, Plate XXVII) and elongated types of nuclei intermediate between this division form and the typical round nucleus were found (Figs. 21 and 22, Plate XXVII). In some of the parasites the protoplasm was also seen to be forked. In the smaller forms this was possibly due to amoeboid action (Figs. 31 and 32, Plate XXVII), but in the larger forms it was sometimes accompanied by elongation or commencing division of the nucleus (Fig. 22, Plate XXVII).

The 'cross-forms' of division characteristic of the genus *Nuttallia* were found on two occasions (Figs. 25 and 26, Plate XXVII).

'Division rosettes' consisting of four small parasites were found in smears from the blood, spleen and bonemarrow (Figs. 27—29, Plate XXVII) and on one occasion a rosette of five was found (Fig. 30, Plate XXVII.)

Galli-Valerio described pairs of piriform parasites occurring in his specimens 'exactly like *Piroplasma bigeminum* and *Piroplasma canis*,' but in spite of a careful search, I have never found such forms, and Yakimoff also noted the absence of such forms from his cases. It would seem that Galli-Valerio was either dealing with a different parasite of the genus *Piroplasma* (*Babesia*) or that his case was a mixed infection with parasites of both the genus *Piroplasma* and the genus *Nuttallia*.

From the above description it will be seen that the parasite under consideration belongs to the genus *Nuttallia*, Franca, 1909, showing the large and small round forms, the large and small oval forms, the large and small rod-shaped forms, the 'cross-form' of division and the 'division rosettes' of four characteristic of that genus.

## PATHOGENICITY.

On account of the resemblance of this parasite to *Nuttalli equi* the parasite of equine piroplasmosis it was important to ascertain the infectivity of this parasite because, if the two parasites were found to be identical, the hedgehog might be acting as a reservoir for infections with *Nuttalli equi*. Yakimoff injected infected blood from a hedgehog into a foal, a pup, two rabbits, two white mice, a field mouse, two porpoises and two doves with negative results.

EXPLANATION OF PLATE XXVII

- Fig. 20. Free form of parasite.
- Figs. 21 and 22. Showing elongation of the nucleus prior to division.
- .. 23 and 24. Showing division of the nucleus into two parts.
- .. 25 and 26. " Cross-forms " of division.
- .. 27 to 30. " Division rosettes."
- .. 31 and 32. Showing early division of the protoplasm or possibly amœboid action.
- .. 33 and 34. Amœboid forms.
- Fig. 35. Intermediate-sized parasites with central nuclei.
- Figs. 36 and 37. Cells containing multiple parasites.

# EXPLANATION OF PLATE XXVII.

- Fig. 30. Free form of parasite.  
 Figs. 31 and 32. Showing elongation of the nucleus prior to division.  
 " 33 and 34. Showing division of the nucleus into two parts.  
 " 35 and 36. " Cross-forms " of division.  
 " 37 to 39. " Division together."  
 " 31 and 32. Showing early division of the protoplasm or possibly amoeboid action.  
 " 33 and 34. Amoeboid forms.  
 Fig. 35. Intermediate-sized parasites with central nuclei.  
 Figs. 36 and 37. Cells containing multiple parasites.

# PLATE XXVII

1676



OUTYALLIA - NORTON



Yakimoff found ticks belonging to the genera *Rhipicephalus* and *Dermacentor* as ectoparasites on the hedgehogs he examined and, from some transmission experiments which he made, thought that the nymphs of *Dermacentor reticulatus* might be the possible carriers of the infection in hedgehogs in Russia.

Galli-Valerio thought that in Tunis it might possibly be carried by *Amblyomma variegatum*. No ectoparasites were found on the two infected hedgehogs examined by me, but this might have been due to such parasites having left the dead bodies, which were not seen by me unfortunately until some hours after death had taken place.

My thanks are due to the London School of Tropical Medicine for affording me facilities for working in the laboratories there and to Dr. J. Gordon Thomson for much help and advice.

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## DESICCATED NUTRIENT MEDIA.

BY

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AND

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[Received for publication, April 12, 1921.]

A NOTE appeared in the *Lancet* by E. T. Thompson (1910) of the Wellcome Physiological Research Laboratories on the desiccation of culture media and the advantages of having at disposal medium which could be rapidly prepared and utilized strictly in the amounts necessary for immediate requirements. The marked success of commercial nutrient medium tabloid, and nutrient medium powders is testimony to the utility of this process. The process as described by Thompson consisted in evaporation of the prepared media to dryness. The same procedure was applied to the ordinary stock medium and to special media alike. Mrs. Norris (1920), working at the Central Research Institute, obtained a digest product from casein which when evaporated to dryness, possessed all the properties and convenience of a nutrient bouillon powder. In our search for means by which we might provide supplies of the necessary materials for bacteriological work ready-made, we have utilized the property possessed by agar-agar and gelatin of imbibing fluid, to enable us to desiccate nutrient media in a comparatively easy manner. The essential point of the procedure was to get a nutrient agar which could be satisfactorily passed through an ordinary meat mincing machine. It was a very easy matter then to dry the vermicular

product which came through the disc of the mincing machine. The mincing renders the whole product particulate and thus enormously increases the free evaporation surface upon which the desiccating process operates. The idea of utilizing minced agar for the purpose has been taken from the published note of Major J. Cunningham, I.M.S., on the desiccation of filtered agar for addition to bouillon in order to solidify it (1919). During the war the subject of provision of bacteriological materials received considerable attention. Uhlenhuth and Messerschmidt (1915) proposed the use of tinned nutrient media, as being particularly suitable for use in the field. A similar method was in use in the Central Research Institute, Kasauli, before the war, and doubtless in other laboratories also, only that the nutrient medium was supplied in the rubber-washed, spring-topped, milk bottles which are used for the transport of sterilized milk. In this way also the media essential for satisfactory work in a bacteriological laboratory can be supplied ready made, and so save the worker the labour of their preparation. But such media are much more bulky and are likewise more liable to spoiling by contamination than a desiccated medium. Thompson, in the article mentioned above, desiccated his special media after they had been prepared. Our object is to supply only a desiccated ordinary nutrient agar or gelatin and to make up special media by first mixing the special ingredients, if possible, in the dried form with the dried nutrient powder and then bringing them all into solution together to constitute a special medium. In this preliminary discussion no mention has been made of a dried nutrient bouillon. The dried nutrient agar can, however, be utilized to prepare a nutrient bouillon. In that case the agar has to be rejected, being utilized only to facilitate the desiccation of the bouillon.

1. METHODS OF PREPARATION.—1.1. Nutrient Agar Powder. (1) Use a tryptic digest of mutton bouillon with reaction p. H. 8 as basis for the preparation of nutrient medium. (2) Cut up agar fibre into small pieces. (3) Add to the bouillon to the extent of 6 per cent. by weight. (4) Place the mixture so prepared for one hour in the autoclave at 120 C. to thoroughly melt the agar. (5) Filter the bouillon agar so obtained, in the autoclave, through cotton wool and muslin, into a receptacle. (6) Cut the agar out of the receptacle and into slices. (7) Pass the slices through a meat mincing machine with a finely perforated outlet disc. (8) Spread the minced nutrient agar on metal or other type of trays. (9) Dry in a hot air oven<sup>4</sup> or in any other

way which is rapid and convenient. (10) Store the powder<sup>5</sup> obtained in a sterile glass stoppered bottle.

NOTES.—<sup>1</sup> Mutton was what we experimented with; beef, or perhaps better still casein, would be still more suitable. <sup>2</sup> Various percentages (by weight) were used:—4, 6, 8 and 10 per cent. The 4 per cent addition afforded a gel which was rather soft for mincing. The 8 and 10 per cent gels were difficult to filter. <sup>3</sup> The reaction now is p.H. 7·4 to 7·6. <sup>4</sup> The oven used by us was one in which heated air passed over metal trays and out through a flue. In the flue the temperature reached about 100C. The process then was not one of drying only but also of dry heat sterilization. Desiccation could be effected at a lower temperature and vacuumization combined with heat. <sup>5</sup> The coarse flaky powder may be made finer by grinding.

1·2. Nutrient Agar Medium. (1) Add 4 to 6 per cent<sup>1</sup> by weight of nutrient agar powder to water in test tubes or flasks. (2) Keep the T.T. for 1 to 2 hours in boiling water or in a steam sterilizer to bring the medium into solution, and to sterilize. (3) Slope the test tubes.

NOTES.—<sup>1</sup> This was the percentage which after trial of various strengths was found to be most satisfactory.

1·3. Nutrient Bouillon (1) Add 6 per cent by weight of nutrient agar powder to cold water (2) Extract at room temperature for 2 hours. (3) Filter. (4) Sterilize the filtrate and use as nutrient bouillon.

1·4. Neutral Red Lactose Bile Salt Agar.<sup>1</sup> (1) Prepare:—sodium taurocholate 0·5; nutrient agar powder 4; distilled water 100. (2) Effect solution in the steamer. (3) Prepare fresh a 1 per cent neutral red. (4) Add to the fluid sodium taurocholate agar 0·2 to 0·5 per cent of the neutral red solution, and 1 per cent lactose. (5) Effect solution<sup>2</sup> of the lactose by gentle heating. (6) Distribute in test tubes. (7) Sterilize 20 minutes at 100C on each of three successive days.

NOTES.—<sup>1</sup> This medium is taken as an example of the preparation of special medium. <sup>2</sup> The lactose may be added in the form of a strong solution and may be sterilized separately for addition to the medium after it has been sterilized.

2. COMPARATIVE NUTRIENT CHARACTER.—The nutrient character of the prepared medium was gauged by taking a rough measurement (length and breadth) of the growth area, suspending the growth in a volume of fluid equal in c.c. to the number of square centimetres of growth and estimating from the turbidity of the suspension the bacterial content of the suspension. This bacterial content is expressed as milligrammes of dried bacterial substance per c.c. The comparison was

made between dried nutrient agar and a tryptic agar made from nutrient. The results were as follows:

TABLE

*Showing yield in milligrammes of dried bacterial substance per square centimetre of growth surface.*

Organism.	Nutrient agar powder.	Ordinary tryptic agar.
<i>B. typhosus</i> ..	1.1	1.2
<i>V. cholerae</i> ..	2.4	2.1
<i>S. aureus</i> ..	2.5	2.5
<i>B. dysenteriae</i> (Shiga)	0.4	0.6

There is no evidence as judged by yield of growth that there is any loss of nutrient quality by a nutrient medium undergoing the treatment here described.

3. SUMMARY AND DISCUSSION. 3.1. The utilization of agar or gelatin to absorb nutrient fluid medium, with subsequent mincing greatly facilitates the desiccation process which reduces the medium to powder.

3.2. A nutrient *fluid* medium can be prepared from the nutrient agar powder by extracting with water in the cold and rejecting the undissolved agar.

3.3. The agar which is utilized to absorb nutrient fluid is itself an essential constituent of solid medium.

3.4. The desiccated medium is as nutrient when made up as the same medium prepared without desiccation.

3.5. The advantages of a desiccated nutrient medium are, 3.51 portability; 3.52 preservability; and 3.53 readiness for use.

3.6. With the solution of the difficulty of provision of large quantities of nutrient medium in small bulk it may be said that the problem of the portability of laboratory outfit is practically solved. The special materials required for special media can be supplied in their natural dry state for ultimate incorporation with simple dried medium.

3.7. The agar method might also be applied to other laboratory supplies which are primarily in a fluid condition. It is doubtful however whether there would be much advantage in applying the

method to the desiccation of high titre agglutinating and hæmolytic sera which can be preserved and supplied in sealed capillary tubes in quantities which serve for single tests.

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# STUDIES IN THE VALUE OF THE WASSERMANN TEST.

## No. IV.

### SIGNIFICANCE AND VALUE OF A POSITIVE WASSERMANN REACTION IN TUBERCULOSIS.

BY

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[Received for publication, April 12, 1933.]

THE sera of 70 patients suffering from tuberculosis were examined by the Wassermann test with the object of finding out if this disease was responsible for a positive reaction. They were all Indian male adults, between the ages of 20-50 years: some of them were in-patients of King Edward Sanatorium, Dharampur and the others belonged to the Hardinge Hospital, Dharampur. Of these 70 cases, 40 were cases of pulmonary tuberculosis, 14 were cases of tuberculosis of the lymphatic glands, and 16 were cases of abdominal tuberculosis. Fourteen out of these 70 cases gave a positive Wassermann reaction as shown in the following table—

TABLE

*Showing the type of the disease and the results of Wassermann test.*

Type of disease.	NUMBER OF Doses OF COMPLEMENT DEVIATED IN THE WASSERMANN REACTION.				ASSEMBLED RESULTS IN WASSERMANN REACTION.		
	0	3	5	8	Positive.	Negative.	Total
Pulmonary Tuberculosis..	32	0	3	5*	8	32	40
Tuberculosis of the Lym- phatic Glands ..	11	1	1	1	3	11	14
Abdominal tuberculosis ..	13	0	1	2	3	13	16

The percentage of positive results was thus 20 per cent.

\* 4 out of 5 cases gave a definite history of recent syphilitic infection.

Buhman (1916) found that 2 out of the 35 tubercular cases he examined gave a positive Wassermann reaction, and these two cases gave a definite history of syphilis.

2. The technique employed throughout this investigation is the same as "Method Number Four" described in the Medical Research Committee Report (1918), the only difference being the inclusion of further controls as described in my paper (Study<sup>2</sup> No. 1, 1919).

#### SUMMARY.

In view of the fact that an unselected, apparently healthy, Indian male adult population has given me a positive Wassermann reaction in 88 cases out of 400, or 22 per cent (Study<sup>2</sup> No. 1, 1919), I infer that a figure of not more than 20 per cent occurring in an unselected Indian male adult population suffering from tuberculosis, must be due to the existence in this population of latent or clinically inactive syphilis.

#### CONCLUSION

Tuberculosis is not a cause of positive Wassermann reaction.

I wish to express my thanks to Lieut.-Colonel W. F. Harvey, C.I.E., I.M.S., Director, Central Research Institute, Kasauli, for advice and criticism, and also to Dr. Nanavate, Medical Officer, King Edward Sanatorium, Dharampur, and to Dr. Kumar, Medical Officer, Hardinge Hospital, Dharampur, for placing facilities at my disposal during this investigation.

*Note.* A number of cases of relapsing fever occurred in Kasauli during the months of October and November of 1920 and I was able to examine the sera of 15 cases. Twelve of these were Indian male adults between the ages of 20-40 years and the remaining three were children between the ages of 1 and 3 years. In all these cases, films were examined for the spirillum before taking the blood for the Wassermann reaction and the findings were positive in all the cases. Ten out of the twelve adults and all the three children gave a positive Wassermann reaction.

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- <sup>1</sup> BUHMAN, R. . . 1916. Specificity of the Wassermann Reaction as quoted in *Medical Research Committee Report*, No. 21, pp. 10-11 (1918).
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SOME NOTES ON CONORHINUS  
RUBROFASCIATUS;  
(DE GEER).

BY

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(Received for publication April 7, 1931.)

This insect has not hitherto been reported in houses in India either in the larval or nymphal stages, though a few adult specimens have been captured inside houses. It has been stated that these adult specimens were attracted to light in the night time from outside.(1)

While searching in Assam for bedbugs, lice and other biting insects in houses infected with kala-azar or otherwise, I came across the first male specimen of *Conorhinus* in a bed of a patient suffering from fever. Since then I have been able to get specimens of different stages of this insect inside houses only, in various villages of that area (Palas Bam). Therefore the presence of this insect inside houses is not merely accidental.

The following table gives detailed information of various catches made of these specimens in houses in different villages of the infected area.

It will be seen that all the specimens of *Conorhinus* recorded in the Table have been found in the following situations inside a house :—

1. Beds of a rush mattress.
2. Cracks in a wooden bedstead.
3. Walls.
4. Pillars supporting a thatch,

5. Corners of a house.
6. Old boxes full of cockroaches,  
and
7. Pigeons' nests.

It will be again evident from the table that the numbers caught each time varied from one to six, but on one occasion, however, as many as sixteen were secured in a single catch. The table will also show that the distribution of *Conorhinus* is not confined to infected houses alone, but that they are also found in uninfected ones.

TABLE

*Giving information of the distribution of* *Conorhinus rubrofasciatus* *in houses of different villages in area infected with kala-azar.*

Name of a village.	Where found in a house.	House infected or uninfected.	Date of capture.	Adult male or female.	Nymph	Larva.
Ajhara ..	Bed of a patient ..	Infected	15-11-20	Male	..	..
Do. ..	Bed ..	Infected	3-12-20	One male	..	..
Do. ..	Bed ..	Infected	4-12-20	One female	2	..
Do. ..	On a pillar in a house ..	?	5-12-20	One female	..	1
Do. ..	In a crack of a wooden bedstead	Uninfected	31-12-20	....	1	..
Gossainpara ..	On a wall ..	Infected	12-1-21	....	2	2
Devripara ..	Bed, specimen found dead ..	Infected	14-1-21	One male	..	..
Sual Kuehi ..	On a wall ..	Infected	16-1-21	....	1	2
Sodilapur ..	On a wall behind a door ..	Infected	21-1-21	One female	..	..
Kaon Bari ..	Pigeon's nest ..	Infected	23-1-21	One male	1	1
Sodilapur ..	In a box full of cockroaches ..	Infected	28-1-21	Four females	10	2
Ranjur ..	In a box containing cockroaches ..	Infected	29-1-21	....	1	..
Dharapur ..	Pigeon's nest ..	Infected	10-2-21	One male	3	2
Gauhati Sadar ..	Old basket ..	Uninfected	21-2-21	....	2	..
TOTAL ..	....	....	..	Males .. 5 Females .. 7	23	11

Most of the specimens were dissected and found to contain ingested mammalian blood. One or two specimens, however, showed non-mammalian blood in their stomach.

The finding of *Conorhinus rubrofasciatus* in houses in a kala-azar area and containing ingested mammalian blood has a significance which is obvious and suggestive. Donovan in Madras had, however, suggested *Conorhinus rubrofasciatus* as a possible carrier of kala-azar, but it was then pointed out that neither the adult insect nor any of its early stages were found in houses.(1).

NOTE:—Since writing the above, I was able to find conorhins in another heavily-infected area in Assam, namely, Dudnai in the District of Goalpara. I could not, however, find any of these insects in the Subdivision of Jorhat which is supposed to be free from kala-azar.

As for the number of specimens caught in a single house, as many as fifty, including larvæ, nymphs and adults were secured in a single catch.

#### REFERENCE.

- <sup>1</sup> PATTON and CRAIG . . Text Book of Medical Entomology, 1913, pp. 488-489.

# THE GEOGRAPHICAL DISTRIBUTION OF THE INDIAN RAT FLEAS AS A FACTOR IN THE EPIDEMIOLOGY OF PLAGUE: PRELIMINARY OBSERVATIONS.

BY

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[Received for publication, 20th April, 1921.]

THE geographical distribution of plague in India has not as yet been fully explained. If we consider, from the facts now known regarding the etiology and epidemiology of the disease, what might have been expected to be the history of the invasion from the time of the introduction of plague at Bombay in 1896, we find a very marked divergence between the theoretical conclusions and the actual course of events. Everywhere in India there are rats, and all the rats harbour fleas; practically all parts of the country have, for a part of the year, a climate favourable, more or less, for the establishment and spread of plague; communications are free, and there are no considerable natural barriers. It would have been reasonable to forecast that the disease would spread from Bombay as from the apex of a fan, until it reached the mountain ranges which separate India from the rest of Asia: that the progress would be on the whole a steady wave but with a more rapid advance along the main lines of communication and where the climatic conditions were the most favourable for the greatest length of time in each year; it would not have been reasonable to forecast that any part of the country would escape.

Within a few years of the first introduction of the disease it spread in all directions, but, in spite of the widespread nature of the epidemics,

large tracts of country escaped altogether, or suffered very lightly. The inequality of the distribution of the disease is very strikingly brought out in the map accompanying the annual Report of the Sanitary Commissioner with the Government of India for 1917. This map shows that the southern portion of the Madras Presidency, east and west of Cape Comorin, large tracts of the eastern coastal area, of the Central India Plateau, and of Eastern Bengal and Assam, and the western half of Burma, have remained free from the disease, although they are near to, and in free communication with, regions which have experienced severe epidemics. Other tracts of country, as for instance that to the west of the Indus, and that on the eastern side of the Madras Presidency, are shown as having suffered little from plague. Although in a general way the epidemics have been most severe in those parts in which the conditions are, according to our present knowledge, most favourable, there are many striking exceptions. The Madras Presidency offers many striking instances of places in which, though the conditions are apparently favourable, the disease has never become established.

It is the absolute failure of the disease to appear in certain areas which is the striking fact. One would not expect the mortality from plague to be evenly spread over the whole country; as the Plague Research Commission have pointed out, the rat population is denser in some parts than in others, and the number of fleas per rat is a variable quantity; climatic conditions may be more or less favourable to the rat flea in its rôle as the vector of plague; free communications may facilitate, lack of communications may hinder, the spread of the infection. But it may safely be affirmed that there is practically no part of the country where the known conditions are definitely and permanently against the establishment of an epidemic. The escape of large and populous tracts throughout a long period of years cannot be explained on the facts as at present known.

The Plague Research Commission paid special attention to this problem of the inequality of distribution. In the case of Eastern Bengal and Assam they expressed the opinion that the physical features of the country, which is bounded on the north and east by mountain chains, and is subject to annual floods, would tend to protect it from the importation of the disease, and to limit its spread if introduced. They found that rats are much less abundant in the houses of the people than in other parts, the houses being less fitted to afford shelter to these

vermin, and food supply for them being less abundant on account of the tidier habits of the people. In the case of the United Provinces, contrasting the heavily infected city of Cawnpore with Lucknow, only forty-five miles away and presenting apparently similar conditions, but showing a much lighter infection, they lay stress on the fact that Cawnpore is a large and important trade centre, with a large trade in grain. Contrasting the comparatively plague-free district of Bundelkhand with the severely infected Ballia district, they found that the climatic conditions of Ballia were the more favourable, and considered that the wandering habits of the people rendered the introduction of the disease more likely. Their conclusions with regard to the irregularity of the distribution of plague in the Madras Presidency are less definite. They lay stress on the importance of the physical features of the country and of the climate, and on the proximity of the infected areas of the Presidency to heavily infected areas in other administrative divisions: they also point out that the more humid and cooler coastal regions, which have generally escaped, are separated from the infected parts by a low-lying and comparatively hot and dry plain, across which infection would not so easily pass.

It may be noted that this irregularity of distribution is to be observed within smaller tracts as well as in the country as a whole. It has been pointed out that the villages within any district of the Punjab are not all equally infected, and that the inequality is not necessarily related to the freedom or paucity of communications.

It is not proposed to enter into a discussion or criticism of the findings or conclusions of the Plague Commission. The writers themselves were clearly not satisfied that they had come to the root of the matter, and do not hesitate to express their doubts. In discussing the distribution of the disease in the United Provinces, after stating in general terms the conditions, or combinations of conditions, affecting the distribution of the disease, they go on to say 'It may well be, however, that other unexplained factors are at work, without knowledge of which a completely satisfactory explanation of the geographical distribution of plague cannot be arrived at.' They came to the conclusion that in Madras City, which has remained practically free from plague, the conditions as regards house construction, etc., are not unfavourable, and that suitable climatic conditions prevail during the winter months. Their later work on the distribution of the disease in the Madras Presidency as a whole did not disclose any very definite explanation as

to why the city should have escaped. It is admitted that plague has been introduced there many times.

We may, therefore, not unreasonably presume that there is an important factor, not yet recognized, in the combination of factors which govern the epidemiology of plague. It is the purpose of this communication to record preliminary observations which indicate that this missing factor is the geographical distribution of the species of *Xenopsylla*.

Throughout their work the Plague Research Commission assumed that there was only one species of this genus on rats in India, *eximius cheopis*. According to their reports on the examination of very large numbers of rat fleas in different parts of the country, this species made up 98 per cent or more of the total, and was therefore the only species of any importance. This, however, is not the case. There are three species, *astia*, *brasiliensis*, and *cheopis*; of these, as will be shown presently, *astia* and *cheopis* are equally common and widespread.

It was pointed out by L. F. Hirst in 1913 that *X. astia* is the common rat flea of Colombo. He recorded also that the whole of a collection of rat fleas which he obtained from Madras, sent by him to Rothschild, were identified as *astia*. Hirst drew attention to the connection between these observations and the relative immunity of Colombo and Madras from Plague, and suggested that further investigations into the geographical distribution of the two species might throw light on the epidemiology of the disease in Madras and Burma. In a further communication in which an account of the plague outbreak in Colombo was given by Phillips and Hirst, certain suggestive peculiarities in the habits of *astia* were noted. From the entomological point of view the suggestion that the several species of the genus, or of other genera, should not be equally efficient as vectors of the disease, is a very reasonable one. We know that parasitic insects differ widely in their host preferences, feeding habits, in their reactions to temperature and humidity conditions, and in their powers of resistance to an unfavourable environment. There may easily be some factor or factors in the bionomics of one or other of these species which may render it practically ineffective as a vector of plague. If that is so, the geographical distribution of the several species is a factor of prime importance in the epidemiology of the disease.

*Material and Technique.*

At the writer's request arrangements were made by the Public Health Commissioner with the Government of India, and by the Sanitary Commissioners of Provinces, for the collection of rat fleas in the Punjab, the Madras and Bombay Presidencies, Central India, and in Burma. Altogether 107 collections were received, representing heavily infected, lightly infected, and plague-free areas. The first collections were received in November, 1919, and the work was continued during the following year. The total number of fleas examined was 17,358.

It is practically impossible to make out the distinguishing features of these species unless the specimens are suitably prepared. The females can, on account of the characteristic shape of the spermatheca, be recognized with a hand lens after the soft parts have been dissolved out by caustic potash or rendered transparent by a clearing agent, but for the certain identification of the males the use of the compound microscope is necessary. The fleas were therefore mounted in the following way. After a short preliminary treatment with caustic potash solution they are brought through alcohol to xylol, and allowed to remain overnight in a thin solution of balsam in xylol. The slides are prepared by coating them with a thin layer of balsam, and allowing them to dry overnight in the incubator. The fleas are then picked up with forceps and laid, similarly orientated throughout, on the slide, in regular rows of ten, five such rows to a slide of ordinary size. The coverslip is moistened with xylol and lowered gently on to the slide. With a little practice the fleas can be arranged as regularly as a series of sections. This method of mounting, which can be done by a trained assistant, saves a great deal of time in the subsequent examination, for the rows can be passed rapidly across the field of the microscope by the mechanical stage; it has the obvious advantage that specimens can be easily marked for future reference.

*Statement of the Results.*

Detailed statements of the results, arranged according to Provinces are given in the accompanying tables. The following summary shows that *astia* and *cheopis* are both common fleas in the parts of India covered by this investigation.

TABLE I.

*Summary of the Results of the Examination of 17,339 Rat Fleas*

	XENOPSYLLA.			<i>Ceratophyllus</i>	<i>Leptopsylla morsitans</i>	TOTAL
	<i>asia</i>	<i>brasiliensis</i>	<i>cheopis</i>			
Punjab ..	2,017	3	3,386	282	..	5,688
Bombay ..	547	849	3,786	1	..	5,183
Central India ..	6	202	72	..	..	280
Madras ..	2,122	280	1,938	84	67	4,491
Burma ..	1,549	..	148	..	..	1,697
TOTAL ..	6,241	1,334	9,330	367	67	17,339
PERCENTAGE ..	36.0	7.7	53.9	2.1	0.3	..

It will be noted that the 19 specimens of *Ctenocephalus felis* and *Echidnophaga sp.* are not included in the above table, and have not been taken into account in calculating the percentages in the detailed statements.

The majority of the collections were obtained from towns of considerable size, a few from villages. It will be noted that the differences between the several collections from one district are often considerable.

TABLE II.

## Punjab.

District	Locality	Date	PERCENTAGE OF EACH SPECIES				Actual Total	<i>Cteno- cephalus felis</i>
			XENOPSYLLA			<i>Ceratophyllus punjaben- sis</i>		
			<i>asiai</i>	<i>brasili- ensis</i>	<i>cheopis</i>			
Ambala ..	{ Barar } { Racheri }	Jan. *	..	..	100	..	6	..
Ferozepur ..	Ferozepur	Dec. 1919	18.8	..	81.2	..	96	..
	Do. ..	Do.	16.1	..	76.8	7.1	99	..
	Do. ..	<b>Total</b>	<b>17.4</b>	..	<b>79.0</b>	<b>3.6</b>	<b>195</b>	..
Gurgaon ..	Gurgaon ..	Dec. 1919	60.8	..	31.1	8.1	74	..
	Hodal ..	Aug.	57.5	..	42.3	..	298	..
	Sukris ..	Do.	100	..	..	..	400	..
	Firozpur ..	Do.	..	..	..	..	..	..
	Jhirka ..	Do.	100	..	..	..	448	..
	Faridabad	Sept.	87.2	..	12.3	..	148	1
	Jacob Pura	Aug.	18.1	..	89.9	..	299	..
	Pulwal ..	Sept.	39.0	..	51.0	..	300	..
		<b>Total</b>	<b>61.6</b>	..	<b>37.9</b>	<b>0.5</b>	<b>1,567</b>	<b>1</b>
Jhang ..	Chisnot ..	Dec. 1919	56.1	(1)†	36.8	6.3	173	..
Jhelum ..	Jhelum ..	Nov. 1919	..	..	86.3	13.7	116	..
Jullundur ..	Jullundur	Nov. 1919	10.0	..	75.3	14.7	150	..
	Do. ..	Do.	37.5	..	37.5	25.0	8	..
	Do. ..	Feb.	6.6	(1)	77.0	16.1	366	..
	Do. ..	April	12.3	..	62.3	25.4	166	..
	Dialpur ..	Jan.	..	..	98.0	2.0	100	..
		<b>Total</b>	<b>7.6</b>	..	<b>77.0</b>	<b>15.4</b>	<b>730</b>	..
Karnal ..	Karnal ..	Jan.	(1)	..	97.4	0.8	119	..
	Do. ..	Mar.	..	(1)	86.1	13.2	130	..
	Do. ..	May	..	..	100	..	148	..
	Do. ..	<b>Total</b>	<b>0.3</b>	<b>0.3</b>	<b>94.7</b>	<b>4.7</b>	<b>397</b>	..

\* When the year is not stated it is understood to be 1920.

† The inclusion of a figure in brackets signifies that it stands for an actual number, not a percentage.

TABLE II -cont'.

District	Locality	Date	PERCENTAGE OF EACH SPECIES				Actual Total	<i>Cteno- cephalus pilosus</i>
			XENOPSYLIDAE			<i>Cteno- cephalus pennsylvan- icus</i>		
			<i>astor</i>	<i>brasil- ensis</i>	<i>chip- pis</i>			
Lahore	Bughia a ..	Nov. 1919	55.1	..	38.5	6.4	176	..
	Kalan Kasur ..	Feb.	2.8	..	95.2	2.0	450	..
	<b>Total</b>		<b>17.6</b>	..	<b>79.2</b>	<b>3.2</b>	<b>626</b>	..
Ludhiana	Ludhiana	Nov. 1919	5.4	..	93.2	(1)	74	..
Lyallpur	Lyallpur ..	Nov. 1919	64.3	..	26.2	9.5	42	..
	Do. ..	Dec. 1919	55.3	..	32.6	7.1	210	..
	Do. ..	Do.	18.3	..	73.9	7.8	299	..
	Gopra ..	Dec. 1919	40.2	..	59.8	..	92	..
	<b>Total</b>		<b>37.6</b>	..	<b>58.7</b>	<b>3.7</b>	<b>643</b>	..
Multan	Darya Khan	Jan.	44.2	..	35.5	20.3	113	..
	Do. ..	Mar.	58.7	..	37.1	4.2	70	..
	<b>Total</b>		<b>50.0</b>	..	<b>35.8</b>	<b>14.2</b>	<b>183</b>	..
Multan	Multan ..	Nov. 1919	100	..	..	..	40	..
	Do. ..	Jan.	99.5	..	0.5	..	210	..
	<b>Total</b>		<b>99.6</b>	..	<b>0.4</b>	..	<b>250</b>	..
Muzaffargarh	Abpur ..	Mar.	13.6	..	84.4	..	22	..
	Muzaffar- garh ..	Dec. 1919	66.7	..	33.3	..	14	..
	Sitpur ..	Mar.	48.0	..	52.0	..	50	..
Rawalpindi	Rawalpindi	Nov.	..	..	92.7	7.3	55	..
	Do.	..	..	..	92.7	7.3	..	..
	Do.	Dec.	..	..	88.0	12.0	50	..
	<b>Total</b>		..	..	<b>90.6</b>	<b>9.4</b>	<b>105</b>	..
Rohtak	Rohtak ..	Jan.	12.8	..	87.2	..	78	..
	Do. ..	April	23.2	..	76.8	..	250	..
	<b>Total</b>		<b>20.5</b>	..	<b>79.5</b>	..	<b>328</b>	..
Sialkot	Sialkot ..	Nov. 1919	3.6	..	85.3	11.1	109	..
	<b>TOTAL</b> ..	..	<b>2,017</b>	<b>3</b>	<b>3,386</b>	<b>282</b>	<b>5,687</b>	..

TABLE III.

Bombay.

District	Locality	Date	PERCENTAGE OF EACH SPECIES				Actual Total	<i>Cteno- cephalus felis</i>	
			XENOPSYLLA			<i>Ceratophyllus</i> sp.?			
			<i>astia</i>	<i>brasil- ensis</i>	<i>cheopis</i>				
Belgaum ..	Athni ..	Nov. 1919	..	..	100	..	111	..	
	Belgaum ..	May	..	55.25	44.8	..	277	1	
		Do. ..	July	..	51.4	48.6	..	300	..
		Do. ..	Aug.	..	33.3	66.7	..	300	..
	Total		..	46.5	53.5	..	877	1	
Bombay ..	Bombay ..	Dec. 1919	49.9	0.5	49.6	..	782	2	
Karachi ..	Karachi ..	Nov. 1919	14.9	..	85.1	..	843	..	
North Kanara	Karwar ..	June	12.5	32.5	55.5	..	40	..	
	Do. ..	Do.	11.2	50.0	38.8	..	18	..	
	Do. ..	Do.	12.2	31.7	56.1	..	41	..	
	Do. ..	July	35.8	11.9	52.3	..	42	..	
	Total		19.1	28.4	52.5	..	141	..	
Poona ..	H. mowar ..	Aug.	..	..	100	..	18	..	
	Poona ..	Nov. 1919	..	12.6	87.4	..	166	..	
		Do. ..	Feb.	..	23.1	76.9	..	337	..
		Do. ..	May	..	22.8	77.2	(1)	350	..
	Do. ..	Aug.	..	11.7	88.3	..	419	1	
Total		..	17.5	82.5	..	1,572	1		
Satara ..	Satara ..	Mar.	..	30.1	69.6	..	390	..	
	Do. ..	Do.	(1)	(1)	95.9	..	49	..	
	Total		0.2	27.3	72.5	..	439	..	
Sholapur ..	Wai ..	July	..	1.0	99.0	..	300	..	
	Sholapur ..	Mar.	3.0	..	97.0	..	100	..	
		TOTAL		54.7	84.9	3,786	1	5,183	4

TABLE IV.

Central India.

Province or State	Locality	PERCENTAGE OF EACH SPECIES				
		Date	XENOPSYLLA			Actual Number
			<i>astia</i>	<i>brasiliensis</i>	<i>cheopis</i>	
Central Provinces ..	Nagpur ..	Mar. ..	..	(1)	95.0	20
Narsingarh State ..	Narsingarh ..	Mar. (1)	5	..	92.5	40
Rewa State ..	Rewa ..	Feb. 27.8	..	..	72.2	18
Do. ..	Umari ..	Jan. ..	..	98.6	1.4	202
		TOTAL	6	202	72	280

TABLE V.

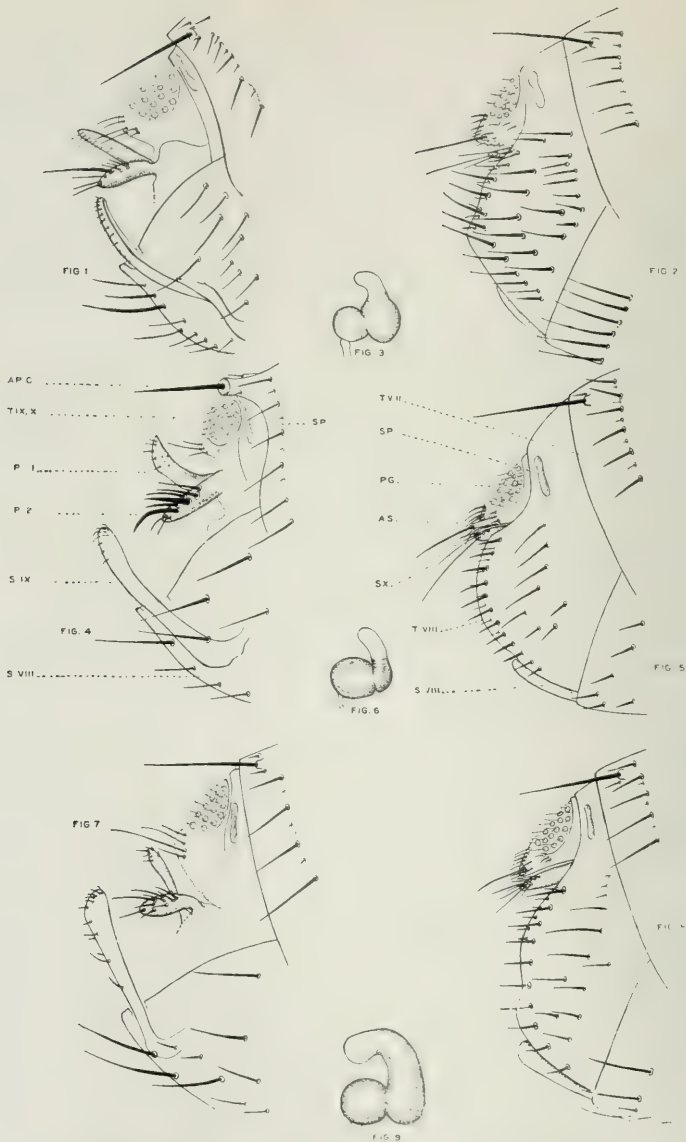
Madras.

District	Locality	Date	PERCENTAGE OF EACH SPECIES					Actual Total	<i>Cteno- cephalus felis</i>
			XENOPSYLLA			<i>Ceratophyllus nigricans</i>	<i>L.lopsylla muscali</i>		
			<i>astia</i>	<i>brasili- ensis</i>	<i>cheopis</i>				
Anantapur ..	Uravakonda	Jan. (1)	..	..	(5)	..	..	(6)	..
Bangalore ..	Bangalore	Mar. 22.7	41.2	..	36.1	..	..	293	..
Bellary ..	Bellary	Jan. 37.5	..	..	62.5	..	..	112	..
	Do.	Do. 39.7	..	..	60.3	..	..	1,000	..
	Do.	May 66.4	..	..	33.6	..	..	300	..
	Hospet	Feb. 44.0	..	..	56.0	..	..	50	..
	Do.	Aug. 15.5	..	..	84.5	..	..	148	..
		<b>Total</b>	<b>42.4</b>	..	<b>57.6</b>	..	..	<b>1,610</b>	..
Gombatore ..	Bhawani	Aug. 91.8	..	..	8.2	..	..	49	..
	Do.	Feb. (1)	(1)	..	96.0	..	..	50	..
	Dharpurm	May 15.6	..	..	84.2	..	..	250	..
	Do.	..	..	..	..	..	..	..	..
	Kokegal	May 6.5	..	54.3	39.2	..	..	40	..
Cuddapah	Cuddapah	Mar. 100	..	..	..	..	..	16	..
	Do.	Oct. 160	..	..	..	..	..	24	..
		<b>Total</b>	<b>100</b>	..	..	..	..	<b>40</b>	..

TABLE V—contd.

District	Locality	Date	PERCENTAGE OF EACH SPECIES					Actual Total	<i>Cteno- cephalus felis</i>
			XENOPSYLLA			<i>Ceratophyllus nilgiricus</i>	<i>Leptopsylla musculi</i>		
			<i>astia</i>	<i>brasil- ensis</i>	<i>chiopsis</i>				
Godaveri ..	Tuni ..	Feb.	..	..	(5)	..	..	5	..
	Cocanada ..	Dec. 1919	77.3	..	22.7	..	..	264	1
Guntur ..	Guntur ..	Jan.	90.6	..	9.4	..	..	149	..
Madras ..	Madras ..	Feb.	100	..	..	..	..	186	..
Nellore ..	Nellore ..	Mar.	80.5	..	19.5	..	..	200	..
Nilgiris ..	Ootacamund	Dec. 1919	..	46.9	35.7	13.2	4.2	98	..
		Do. ..	Do.	..	22.7	36.0	36.0	5.3	97
	Do. ..	Do.	..	22.2	34.6	12.3	30.8	81	..
	Do. ..	Jan.	..	25.0	39.0	15.5	20.5	168	..
	Total		..	28.8	37.2	18.9	15.1	444	..
North Arcot..	Chittoor ..	Feb.	100	..	..	..	..	54	..
Salem ..	Salem ..	Feb.	10.0	..	90.0	..	..	149	1
South Arcot..	Tirukkailur	Sept.	100	..	..	..	..	100	..
	Kallakurchi	June	100	..	..	..	..	100	..
South Canara	Do. ..	Do.	100	..	..	..	..	24	..
	Mangalore ..	Nov. 1919	16.9	3.0	80.1	..	..	166	..
Tanjore ..	Negapatam..	Dec. 1919	100	..	..	..	..	84	..
Trichinopoly	Trichinopoly	Jan.	95.5	..	4.5	..	..	44	..
	Do. ..	Feb.	51.3	..	48.7	..	..	38	..
	Do. ..	Do.	70.9	..	29.1	..	..	62	..
	Do. ..	Do.	90.0	..	(1)	..	..	21	1
Total		74.7	..	25.3	..	..	166	2	
Vizagapatam	Vizagapatam	Jan.	58.3	..	41.7	..	..	36	..
		April	40.0	..	60.0	..	..	20	..
		Total	51.8	..	48.2	..	..	56	..
TOTAL ..		..	2,122	280	1,938	84	67	4,491	3





# PLATE XXVIII

Figures 1 to 10. — *Hydrobia ulina* (L.)  
 1. Antenna of male.  
 2. Antenna of female.  
 3. Antenna of male.  
 4. Antenna of female.  
 5. Antenna of male.  
 6. Antenna of female.  
 7. Antenna of male.  
 8. Antenna of female.  
 9. Antenna of male.  
 10. Antenna of female.

EXPLANATION OF PLATE XXVIII

- Figs. 1, 2, and 3. *X. astra* Rothschild, terminal segments of male,  
female, and the spermatheca.  
4, 5, and 6. *X. brasiliensis* Baker.  
7, 8, and 9. *X. cheopis* Rothschild.

TABLE VI.

*Burma.*

District	Locality	Date	PERCENTAGE OF EACH SPECIES				
			XENOPSYLLA			Actual Numbers	<i>Cteno- cephalus</i> <i>felis</i>
			<i>astia</i>	<i>brasi- ensis</i>	<i>cheopis</i>		
Akyab ..	Akyab ..	Nov. 1919	96.0	..	4.0	50	..
		Do. .. Dec. 1919	100	..	..	20	..
		Do. .. Jan.	84.4	..	15.6	141	..
		Do. .. Feb.	..	..	..	..	..
		Do. .. April	99.4	..	0.6	442	1
		Do. .. July	99.5	..	0.5	599	..
		Do. .. Oct.	100	..	..	163	..
		<b>Total</b>	<b>97.8</b>	..	<b>2.2</b>	<b>1,421</b>	<b>2</b>
Moulmein ..	Moulmein ..	June	70.6	..	29.4	95	..
Rangoon ..	Rangoon ..	Dec.	54.4	..	45.6	136	..
	Do. ..	July	40.0	..	60.0	45	..
<b>Total</b>			<b>50.8</b>	..	<b>49.2</b>	<b>181</b>	..
<b>TOTAL ..</b>			<b>1,549</b>	..	<b>148</b>	<b>1,697</b>	<b>2</b>

*The Three Species of Xenopsylla.*

Descriptions of these three species were given by Rothschild in the Bulletin of Entomological Research in 1914. References to the original more detailed descriptions are given at the end of this paper. *X. cheopis* is the only one of the three which was included in the 'Revision' published in 1906.

The diagnosis of these species depends mainly on the form of the terminal segments of the abdomen. These are illustrated on Plate XXVIII. The writer has seen no deviations from the described types which were sufficient to lead to doubt regarding the identification. The spermatheca of the female is remarkably constant in shape in each of the three species, and furnishes a ready means of distinguishing them.

### 386 *Geographical Distribution of the Indian Rat Fleas.*

Of the large number examined, only two variations were found, both in *cheopis*: in one of these the tail of the spermatheca was very short, giving the organ an L-shape; in the other there were two spermathecae, each of the normal size and shape.

There is one point not mentioned by Rothschild which serves to distinguish the male of *astia* from the males of the other two species. The median sagittal incassation of the head has an even contour in *cheopis* and in *brasiliensis*, but in *astia* it is produced ventrally in its posterior portion, so as to have a 'keel' like appearance. This character may not, of course, be peculiar to *astia*, but it is not present in the other two Indian species.

It should be noted that Rothschild's synopsis refers only to these three Indian species. There are others of this genus in which the antepygial bristle is placed on a raised tubercle, as in *brasiliensis*, and others which have a ribbon-like ninth sternite, as in *astia*.

#### *The Geographical Distribution of the Three Species of Xenopsylla.*

An examination of the tabular statements given, and of the accompanying map, shows at once that the distribution of these species, so far as it is indicated by the scattered and far too scanty observations here recorded, is most irregular, and, in the case of *astia* and *cheopis*, is not clearly correlated with any of the factors which ordinarily govern the geographical distribution of animals. The case of *brasiliensis* is less obscure, and it will be convenient to consider this species first.

Speaking very generally, India consists geographically of two distinct parts: Peninsular India, an elevated table-land, geologically ancient, occupying the central and southern portions; and Extra-Peninsular India, which is mainly composed of the great Indo-Gangetic Plain, a geologically recent tract of country which, though it extends from the coast to the foot of the Himalayas on either side of the Peninsula, is not more than a few hundred feet above sea-level. The habitat of *X. brasiliensis* is clearly Peninsular India. Of the seventeen stations from which it is recorded, only three are in the Punjab, and from each of these only a single specimen was obtained. In the remaining fourteen stations the percentage of this species varied from less than one to nearly 100, with an average of about 29. Only three of these stations are on the coast line, and these, it will be noted, are all on the west coast, where the range of the Western Ghats runs almost down to the sea. The

numerous stations on the east coast of Madras from which batches have been obtained are all far distant from any high land, and have yielded no specimens of *brasiliensis*.

The special characters of the stations which show a considerable percentage of *brasiliensis*, in contrast to the Indo Gangetic Plain and to the coast line on the east, are the absence of extreme ranges of temperature such as are met with in the Punjab, and a moderate degree of humidity. All these stations are in the tropics.

The nature of the conditions favourable to this species is well illustrated by the three batches obtained from the Coimbatore district in Madras. No specimens of *brasiliensis* were present in the 349 fleas obtained from Bhawani and Bharapuram, which are between 600 and 1,000 feet above sea-level, while at Kollegal, in the same district, but at the edge of the Mysore plateau and at the 1,500-3,000 level, more than half were of this species. It will be noted that *Ceratophyllus punjabensis* and *X. brasiliensis* do not occur together.

*X. astia* and *cheopis* occur all over India, equally in the Peninsula and in the Extra-Peninsular portions; their relative prevalence cannot be correlated with climatic conditions, nor does it appear to follow the divisions into which zoologists have mapped out the country from a study of the fauna as a whole. Each species is present in considerable numbers in the sub-tropical regions of the Punjab, where the climate is subject to wide seasonal variations, and on the east coast, where the climate is tropical throughout the year and the humidity always high. Of the forty-two stations from which substantial batches of fleas were obtained, *cheopis* was present in all but five, *astia* in all but eight; it may be noted that in three of the batches from which *cheopis* was absent the total number of fleas examined was small. On the whole, however, it is evident that *cheopis* is the commoner flea in the Punjab, while *astia* is the more common on the Madras coast, taking the two extremes of climate. In some of the stations on the Peninsula *astia* appears to be replaced by *brasiliensis*; all batches which contain *brasiliensis* also contain *cheopis*.

The bearing of climate on the relative prevalence of these two species is indicated in the accompanying map, on which the means of temperature and humidity are shown. The group of Punjab stations having a mean annual temperature of less than 77.5°F. show a high percentage of *cheopis*, but in two of them more *astia* are recorded than *cheopis*; the proportions here appear to be more nearly related to the mean annual

humidity. The group of stations having a mean annual temperature between 77.5° and 79°F. show a distinctly higher proportion of *astia*. The stations on the Madras coast show generally a low percentage of *cheopis*, but at Vizagapatam the proportions of *astia* and *cheopis* are nearly equal. Perhaps the most striking contrast is shown by the three stations in Burma from which collections were obtained. Out of the 1,421 fleas sent from Akyab, only 2.2 per cent were *cheopis*, while at Rangoon, not far distant and with a similar climate, the 181 fleas collected were divided equally between *astia* and *cheopis*. It is clearly not possible, therefore, to correlate the constitution of the flea population with climatic and geographical conditions.

A critical examination of the figures given in the tables shows that the irregularity of the distribution of these two species holds for the smaller areas as well as for the country as a whole. In the Gurgaon district, for instance, a batch from the village of Sakris showed 100 per cent *astia*, while at Jacob Pura, in the same district, the percentage of this species was 18.1, the remainder being *cheopis*. In some instances, two batches collected from the same town during the same month show considerable differences; for example, of the two batches collected at Lyallpur during December, one contained 55.3 per cent *astia*, the other only 18.3. This is probably to be accounted for by the collections having been made in different parts of a large town. The figures suggest, as one would indeed expect to be the case, that, in an area in which both species occur, they are not uniformly distributed over all the rats, but sometimes the one, sometimes the other, will predominate on the rats of particular localities within the area; it is to be expected that batches from adjacent villages will differ within wide limits, when the total collections are small. It is obvious that large collections are essential if statistical methods are to be applied to this problem.

#### *Seasonal Prevalence of these Species.*

The number of large collections obtained at different seasons from the same station is unfortunately too small to allow of conclusions being drawn regarding the seasonal prevalence of these species. The best series is that from Poona. As is shown in the accompanying chart, the proportion of *cheopis* rises from May to September, probably in association with the marked fall in temperature and the increase in humidity which accompany the breaking of the monsoon in June. This suggests that *cheopis* is better adapted to a cool and damp climate than is *brasiliensis*.

a suggestion which is borne out by other considerations already indicated. The figures for Belgaum point in the same direction, though

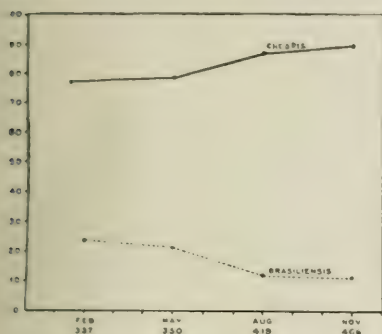


CHART I.—Showing the relative seasonal prevalence of *cheopis* and *brasiliensis* in Poona.

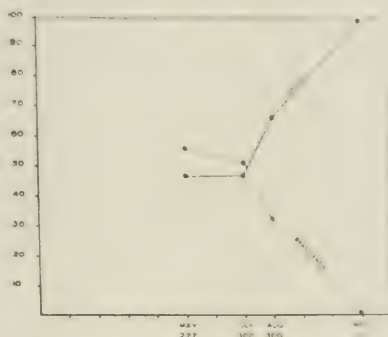


CHART II.—Showing the relative seasonal prevalence of *cheopis* and *brasiliensis* in Belgaum and Athni.

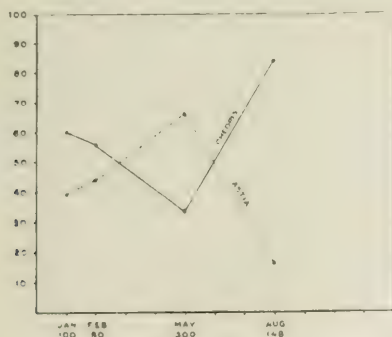


CHART III.—Showing the relative seasonal prevalence of *cheopis* and *astia* in Bellary and Hespel.

The numbers shown below the months are the totals of the collections from which the graphs are constructed.

here it is to be noted that the fleas obtained in November were collected at the town of Athni, not in Belgaum itself. The only station from which successive large batches containing *astar* and *cheopis* were obtained

# 390 *Geographical Distribution of the Indian Rat Fleas.*

is Bellary. Combining the figures for Bellary and Hospet, which is about forty miles distant and in the same kind of country, we find a similar increase in the proportion of *cheopis* during the colder months of the year. Further observations on this point, which is clearly of importance, are required.

## *Proportions according to the Species of Host.*

In few cases has it been possible to obtain precise information regarding the species of rat from which the fleas were obtained. The batches sent from Akyab and from Rangoon were labelled with the name of the host, and the figures are given below. They are sufficient to show that either *astia* or *cheopis* may parasitize any one of several rodents. This is what one would expect from a general knowledge of the relations of fleas to their hosts, and is in agreement with the data given by Jordan and Rothschild.

TABLE VII

*Showing the Host Species of 1,232 Fleas from Akyab and of 181 Fleas from Rangoon.*

				XENOPSYLLA.		TOTAL
				<i>astia</i>	<i>cheopis</i>	
RANGOON.						
N.	<i>bengaliensis</i>	..	..	42	20	62
M.	<i>concolor</i>	..	..	28	57	85
M.	<i>norvegicus</i>	..	..	10	3	13
M.	<i>rattus</i>	..	..	3	1	"
"	Bandicoot	..	..	6	..	6
"	Shrew	..	..	1	..	1
"	Mouse	..	..	2	8	10
TOTAL				92	89	181
AKYAB.						
N.	<i>bengaliensis</i>	..	..	562	5	567
M.	<i>rattus</i>	..	..	266	23	289
M.	<i>concolor</i>	..	..	373	3	376
TOTAL				1,201	31	1,232

*Ceratophyllus.*

Until quite recently it was supposed that the *Ceratophyllus* found on rats in India was the same as that commonly found on rats in Europe, *fasciatus* Bosc. and it is thus referred to in plague literature. There are, however, not one but several species, and of these *fasciatus* is so rare that there is a doubt as to whether it is a true Indian flea. Jordan and Rothschild, in a paper published in January of this year, give eight species of *Ceratophyllus* from rats and squirrels in India, and of these seven are new. They give two records only of *C. fasciatus* in India.

The results of the present investigation confirm the findings of the Plague Research Commission with regard to the distribution of *Ceratophyllus*. With the exception of a single specimen among the 1,572 fleas from Poona, the genus was not represented in the Bombay collections. In Madras *C. nilgiriensis* was found in considerable numbers at Ootacamund, in the Nilgiri Hills and some 6,000 feet above sea-level. In the Punjab, on the other hand *C. punjabensis* was present in the collections from twelve out of the fifteen stations, and in some instances occurred in considerable numbers: three batches from Jullundur contained 14.7, 16.1, and 25.4 per cent.

The marked seasonal prevalence of this species, which was commented on by the Plague Commission, is strongly brought out by the figures from the Punjab. None of the seven batches of fleas collected during the hot weather contained *Ceratophyllus*. It will be noted in Table II that of the seven batches from Gurgaon, collected during August and September, none contained this flea, while the batch collected in December contained 8.1 per cent. If we omit the fleas collected during the hot weather the average percentage of 70 batches is 6.5. The observations of Bacot on the prolongation of the cocoon stage of *C. fasciatus* are particularly interesting in this connection.

*Fleas of other Genera.*

The only other true rat flea found is *Leptopsylla musculi*, which was present in the collections from Ootacamund. Specimens from these batches were sent to Mr Rothschild, who pronounced them to be true *musculi*. The occurrence of this species in the Nilgiri Hills, and not elsewhere, is particularly interesting.

The absence of fleas other than those proper to rats is rather striking in view of the large numbers dealt with. It will be noted that there

### 392 *Geographical Distribution of the Indian Rat Fleas.*

were only ten specimens of *Ctenocephalus felis*, the common flea of dogs and cats in India, and not a single *Pulex*.

#### THE GEOGRAPHICAL DISTRIBUTION OF THE INDIAN RAT FLEAS IN RELATION TO THE EPIDEMIOLOGY OF PLAGUE.

The nature of the problem towards the solution of which the present investigation has been directed has already been indicated at the beginning of this paper. We have now to consider how far the observations here recorded bear out the hypothesis that the several species are not equally efficient as vectors of plague, with the corollary that there is a direct relation between the geographical distribution and relative numbers of these species and the incidence of plague.

The observations so far made are neither sufficiently numerous, nor are they sufficiently evenly distributed. The collections vary greatly in size, and at the best represent only a portion of the country; they have not been made in a manner calculated to eliminate the error due to the uneven distribution of the species in the particular locality which they represent; the factor of a difference in the seasonal prevalence of the species has not been altogether eliminated. On the other hand, the figures for the plague mortality of the area represented by each collection or series of collections are averages, and it is known that the mortality is not very evenly spread over the areas to which the figures refer. Nevertheless, the results are striking, and leave little doubt regarding the importance of the difference in species of the predominant flea in heavily infected and in lightly infected parts of the country.

The only two species of which large numbers have been obtained are *X. astia* and *X. cheopis*, which together make up 90 per cent of the total. The present problem is therefore a comparison of those stations in which *astia* is the common flea with those in which *cheopis* is predominant.

The source of information regarding the incidence of plague in the numerous scattered stations from which collections have been received is the map, already referred to, which accompanied the Annual Report of the Sanitary Commissioner with the Government of India for 1917. In this the distribution of plague in India is shown graphically by different kinds of shading; the variations within small units such as districts are not of course shown. The information obtained from this

## MAP 1.

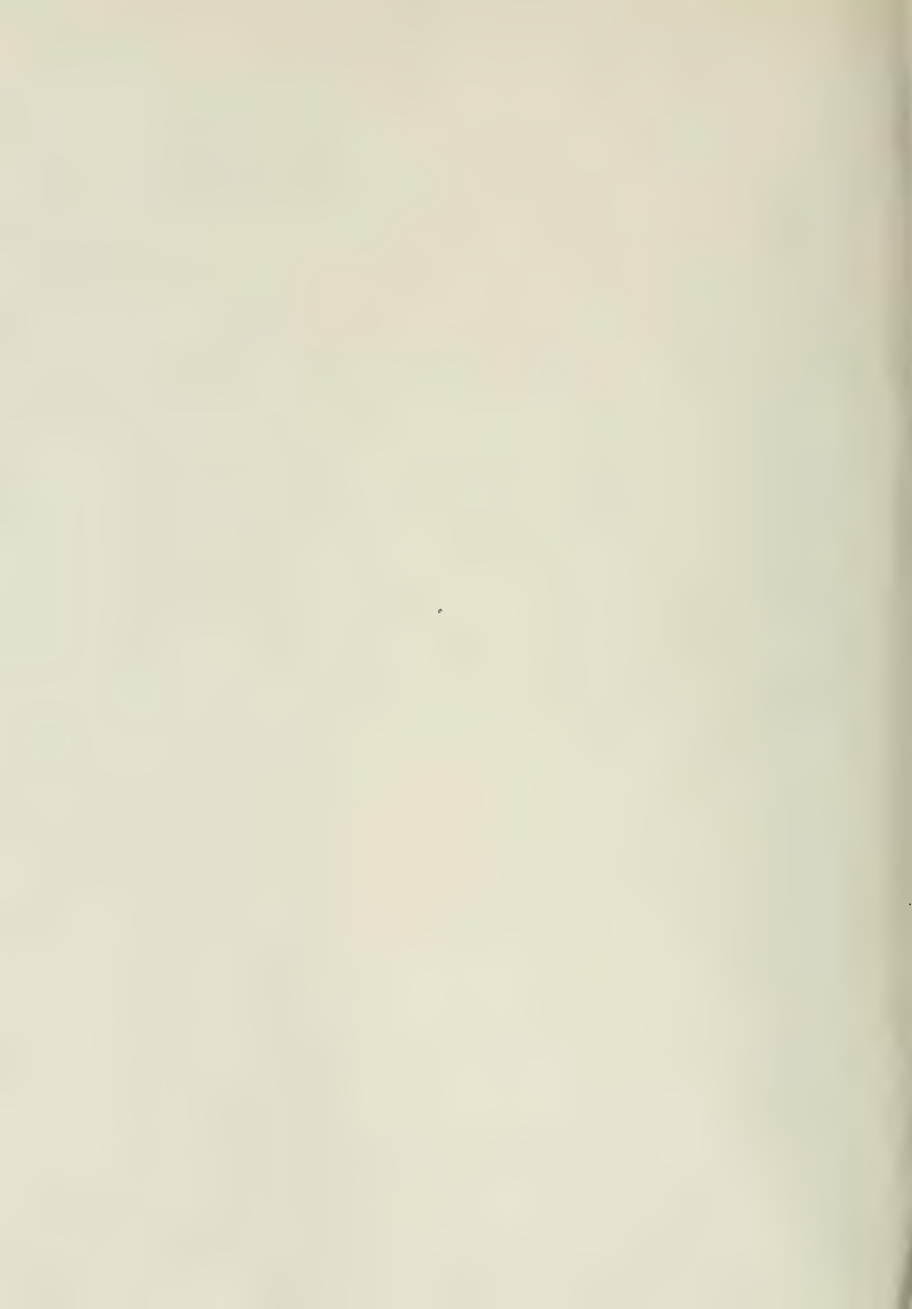


MAP OF INDIA AND BURMA.

*Showing the places mentioned in the text.*

The mean annual humidity (black lines), and the mean annual temperature of Bangalore, 1946 from the Climatological Atlas of India. The interrupted red line is the mean isotherm.

MAYOR, F. W. CRAIG.—The Geographical Distribution of the Indian Rat Flea.





map has been supplemented, through the courtesy of Civil Surgeons and Health Officers of Municipalities, by detailed records from some of the localities from which the fleas were collected.

Omitting places from which small collections were obtained, the localities are arranged in Table VIII according to the percentage of *X. cheopis* and the plague mortality as shown in the above map I. This table should be read in conjunction with Tables II to VI, in which the details of the constitution of each batch of fleas are given. It will be seen that, though the nature of the records is not well adapted for the application of the statistical method by which such a problem should be approached, the observations point very strongly to a close relation between the predominance of *X. cheopis* and high plague mortality. *X. cheopis*, in fact, appears to be truly the 'plague flea,' while *astia* is the predominant species in those areas which have remained free from the disease or have suffered only lightly.

The figures at the two extremes of the table are naturally of the most interest and importance. At Akyab, from which only 31 specimens of *cheopis* were obtained out of a total of 1,421 fleas, there has never been a case of indigenous plague; the collections represent all seasons of the year. At Chinna Salem, in the Kallakurchi Taluk of the South Arcot district, from which 124 specimens of *astia* and none of *cheopis* were obtained, there were two cases of plague in 1911, none before or since. At Guntur, where the proportion of *astia* is 90·6 per cent, there was an outbreak of plague in 1918-1919, with a total of 232 deaths. Special interest attaches to the figures for Madras Municipality, since the conditions there were examined by the Plague Research Commission, who came to the conclusion that they were generally such as would permit of the establishment of the disease. The whole of a batch of 186 fleas were *X. astia*. The plague mortality from 1897 to 1917 has ranged from nil in 1915 to 0·1 in 1906 with an average of 0·013; although the disease has been present in twenty years out of the twenty-one, in thirteen of these years the mortality has been less than 0·01; in other words, though the infection has been repeatedly introduced, it has each time failed to set up an epidemic. At Negapatam, in the Tanjore district, plague was imported in 1918 and in 1919, one fatal case only occurring in each of these years; all of the 84 fleas obtained there were *astia*. At the other extreme we have the very heavily infected districts in the Punjab and in the Bombay Presidency. Unfortunately, no figures for these areas, more exact than

those given in the map, are available at the time of writing.\* The Punjab has throughout been the most heavily infected part of India, and the incidence has been spread fairly evenly over the province, with the exception of the south-western part, including Multan, Muzaffargarh, and Mianwali, which have not suffered severely. The districts named, it will be noted, show a considerable percentage of *astia*. Rohtak and Gurgaon are south-east of the most heavily infected parts. The heavily infected districts in the Bombay Presidency, Wai, Satara and Poona, are all situated at a considerable altitude, and in the two latter stations the collections have included a large proportion of *brasiliensis*.

The liability to severe epidemics of plague in those regions in which *cheopis* is the predominant rat flea, and the absence of such epidemics in regions where *astia* is predominant, seems fairly clear. The observations with regard to *brasiliensis* and *Ceratophyllus* are not sufficient to justify any inference, and their presence in considerable numbers rather complicates the question as between *astia* and *cheopis*. The case of *brasiliensis* can only be dealt with by the examination of a series of collections from Peninsular India, where this species appears to be a common rat flea, and partly to replace *astia*. *Ceratophyllus* is unlikely to be important, as there are few stations in which it makes up a substantial proportion of the whole.

To obtain the more precise data which will justify the full application of statistical methods, it will be necessary to make a series of observations under definitely chosen conditions. Large batches of fleas, collected at the same time of year and preferably during the plague season, are required, and it will be of advantage if only two species are present, either *astia* and *cheopis* or *brasiliensis* and *cheopis*. The stations chosen should be within fairly narrow geographical limits, and the range of plague mortality a wide one. The writer hopes to be able to record such a series of observations shortly.

The great practical importance of the matter is obvious. If it is really the case that *cheopis* is the 'plague flea,' while *astia* is not, it will be possible, by an examination of the fleas of a locality, to estimate precisely its liability to plague; in fact, to map out the '*cheopis* belts,' just as the 'fly-belts' of Africa have been mapped out. It would clearly be unnecessary to take elaborate and expensive measures against plague in a district in which the rat fleas were of a species which is not a vector of plague. The significance of an imported case of plague will depend in a large measure on the local species of flea.

*Acknowledgments.*

The writer is glad to have this opportunity of expressing his most sincere thanks to those who have provided him with the material from which these observations have been made. He is fully conscious of the fact that without the assistance so freely given him nothing could have been done, that, indeed, the present contribution represents to a large extent the work of others. Where so many have been so helpful individual acknowledgments would be rather out of place, but the writer would like to be permitted to express his particular thanks to Chitre, Assistant Surgeon of the Plague Prevention Enquiry, and to his successor, Dr. Strickland, for the large batches, sent regularly at three-monthly intervals, which illustrate the seasonal prevalence of the two species occurring in Poona; and to Major MacWatters, I.M.S., for the large collections from Akyab.

The assistance given by Dr. Jordan and by Mr. Rothschild, who have named the species of *Ceratophyllus* and confirmed the identification of *L. musculi*, is gratefully acknowledged.

## REFERENCES.

- |                                       |    |                                                                                                                                                                                                                                                                                                      |
|---------------------------------------|----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
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398 *Geographical Distribution of the Indian Rat Fleas.*

REFERENCE LETTERING.

- a.s.,      anal style of the female.  
ap.b.,    antepygidial bristle.  
pg.,      pygidium.  
P., P2.   Processes of the clasper.  
S VIII, IX, X. The eighth, ninth, and tenth sternites.  
sp.,      spiracle.  
T VII, VIII, IX, X. The seventh to tenth tergite .

## **NOTICE.**

# INDIAN SCIENCE CONGRESS

1922.

THE 9TH ANNUAL MEETING OF THE INDIAN SCIENCE  
CONGRESS WILL BE HELD AT MADRAS FROM THE  
30TH JANUARY TO 3RD FEBRUARY, 1922.

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THE MEDICAL RESEARCH SECTION OF THE CONGRESS WILL  
THEN MEET FOR THE FOURTH TIME.

*The subscription for membership of the Congress is Rs. 10 - and should be paid to the Honorary Treasurer, Asiatic Society of Bengal, 1, Park Street, Calcutta, who will send a ticket of entry.*

Those who propose to be present at the meeting are requested to communicate, as early as possible, with the undersigned and with Dr. J. L. Simonsen, Forest Research Institute and College, Dehra Dun. The Honorary Local Secretaries are Captain Clive Newcomb, M.D., A.M.C., I.M.S., Chemical Examiner, Madras, and Khan Sahib Mohomad Azizullah Sahib Bahadur, B.A., M.B.C.M., F.M.O., Chemical Examiner's Office, Madras, to whom all enquiries about accommodation should be addressed, Europeans writing to the former, Indians to the latter.

It is essential that notice should be given to the local Secretaries as early as possible.

A notice for the guidance of those who intend to read papers and take part in the discussions will be published in the *Indian Journal of Medical Research* and the *Indian Medical Gazette*.

All papers should be forwarded to the undersigned before the 30th November, 1921. *Each paper should be accompanied by an abstract.*

It will not be possible to include in the programme of the Medical Research Section any paper which has not been sent in to the undersigned by the date mentioned.

The time allowed for the reading of any paper at the Congress will not exceed 15 minutes.

It is hoped that the majority of the papers received will fall into groups which will afford opportunity for discussion. Definite subjects for discussion will be arranged later and proposals for these are invited.

The undersigned will be glad to receive suggestions from any intending member regarding any matters connected with the Medical Research Section.

THE KING INSTITUTE,  
GUINDY, MADRAS,  
*The 26th July, 1921.*

} J. CUNNINGHAM, MAJOR, I.M.S.

## **NOTICE.**

# INDIAN SCIENCE CONGRESS

TO BE HELD AT MADRAS, BETWEEN 30TH JANUARY  
TO 3RD FEBRUARY, 1922.

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### MEDICAL RESEARCH SECTION.

*The President of the section wishes to invite the attention of Members who propose to attend the Medical Research Section to the following points which may be of use to them in the selection of subjects for papers :—*

#### 1.

##### TYPES OF PAPERS DESIRED.

- (1) Announcing in general terms, with no unnecessary detail of description and without complex graphs and lists of figures, any new work performed by the author in any line which has a direct bearing on the advance of medical science and the prevention or treatment of disease.
- (2) Describing, illustrating and demonstrating new methods and technique.
- (3) Briefly summarising present knowledge and indicating promising lines of advance.
- (4) Opening up a discussion on important current medical problems about which there is, or may be, room for divergent opinions.
- (5) Dealing with diseases in animals and plants in so far as they have a bearing on human maladies.

## ILLUSTRATIONS.

Illustrations add greatly to the interest and comprehensibility of a paper, but they should be simple enough or clear enough to be taken in at a glance. Complex diagrams which require close inspection and study divert the attention of the members from the text of the reader. Clear lantern slides are about the best form of illustration, and are suitable for photographs, photomicrographs, graphs and short lists of figures. Line diagrams on a large scale on paper which can be pinned on a screen are also useful. Blackboard sketching or figuring is not recommended.

## II.

## TYPES OF PAPERS NOT CONSIDERED SUITABLE.

- (1) Very detailed and complex, whether in words or figures.
- (2) Very special and outside the range of knowledge of those likely to be present.
- (3) Very general, containing nothing new and putting nothing in a new light.
- (4) Papers which would appeal more to other sections such as the entomological, chemical or botanical.
- (5) Papers on general or clinical medicine, surgery or therapeutics which would be acceptable at a general medical congress.

## III.

## MEMORANDUM.

- (1) Papers should be written in a style suitable for reading rather than with a view to ultimate publication. Minor alterations considered necessary for the latter purpose can be made at a later date.
- (2) No paper should occupy more than 15 minutes in the reading. Members are particularly requested to observe this rule, which is made in their own interests, and they should see beforehand, by actual trial, that their papers can be read without any undue hurry, within the time allotted.
- (3) Discussion will be confined within the following limits :—
  - (a) the production of additional evidence in favour of statements made or suggestion of means to obtain it;
  - (b) the production of additional evidence throwing doubt on or controverting the statements made;

- (c) the questioning of the soundness of the methods used to obtain the results presented, and the suggestion of alternative or sounder methods ;
- (d) asking for the elucidation by the author of obscure passages ;
- (e) supporting or questioning the validity of arguments used .
- (f) supporting or questioning the conclusions drawn from the evidence presented ;
- (g) supplying possible answers to questions raised ;
- (h) raising questions relevant to the subject, but not suggested by the author ;
- (i) the presentation of a rival hypothesis.

Finally, the author of the paper will be given an opportunity of answering all criticisms and summarizing the discussion. No other member may speak more than once, except to correct misinterpretation of anything he may have said.



## KALA AZAR INQUIRY OF THE INDIAN RESEARCH FUND ASSOCIATION.

### ANNOUNCEMENT.

As the January, 1922, Number of the *Indian Journal of Medical Research* was going to Press, a telegram, dated 28th November, 1921, was received from Mrs. Adie, Tezpur, Assam, in which she states :—  
“ Director on tour, so I inform you direct of swarming infection of Leishman-Donovan bodies found in salivary glands and ducts of *C. rotundatus* caught 24th instant on bed of suspected Kala-azar case in infected area. Dissected 26th instant. The discovery positively proves that the bed bug is capable of transmitting the disease through biting.”

Mrs. Adie is working in Assam with the Scientific Committee for the investigation of Kala-azar directed by Major F. P. Mackie, r.m.s., under the auspices of the Indian Research Fund Association.

The Editors decided not to delay publication of the result contained in the telegram, but to issue it at once in the January, 1922, Number of the Journal, as the observation announced is of great scientific interest and, if confirmed, will be a most important addition to our knowledge of the etiology of Kala-azar.



# BACTERIOLOGICAL AND LABORATORY TECHNIQUE.

## SECTION IV.

BY

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*Director, Central Research Institute, Kasauli.*

[Received for publication, May 9, 1911.]

### S 7 STANDARDIZATION.<sup>1</sup>

**Notes.**—<sup>1</sup>A term used here to indicate conceptions such as potency, measurement, titration, standard character, etc.

#### S 7.1 TURBIDITY.

**S 7.11.**—(1) Set up a series of picked T.T. all of the same calibre. (2) Prepare the following solutions:—No. 1, 1 per cent chemically pure sulphuric acid and No. 2, 1 per cent pure barium chloride. (3) Add definite proportions of No. 1 sol. to No. 2 sol. to give standard turbidity suspensions as follows:—99 c.c. No. 1 sol. to 1 c.c. No. 2 sol., 98 c.c. No. 1 sol. to 2 c.c. No. 2 sol., 97 c.c. No. 1 sol. to 3 c.c. No. 2 sol., and so on down to 90 c.c. No. 1 sol. to 10 c.c. No. 2 sol. (4) Place convenient quantities of the resulting suspensions in the picked T.T. and seal off these T.T. in the blow pipe flame. (5) Compare the turbidity of the test suspension placed in a T.T. of the same calibre as those containing the standard suspensions with these suspensions. (6) Find<sup>2</sup> with which suspension in the standard series the test suspension corresponds.

**Notes.**—<sup>2</sup>This is best done by placing each of the standard suspension T.T. in succession alongside the test suspension in a good light over a dark painted surface. The test suspension may require dilution to bring it within the range of the standard series. In that case the degree of dilution must be taken into account in calculating the strength of the test suspension.

**S 7·12** (1).—Precipitate a freshly made strong sol. of barium chloride with excess of sulphuric acid. (2) Boil the mixture. (3) Collect the precipitate on filter paper. (4) Wash the precipitate repeatedly with D.W. to remove all trace of acidity. (5) Dry the precipitate. (6) Complete the drying by roasting the precipitate in a porcelain evaporating dish. (7) Allow to cool. (8) Weigh out very carefully a quantity of the dried barium sulphate. (9) Grind up the dried barium sulphate in a mortar with gradual addition of 1 per cent sod. citrate sol. to make

1 per cent suspension. (10) Prepare from the 1 per cent stock suspension by dilution with 1 per cent sod. citrate a 0·1 per cent barium sulphate suspension. (11) Prepare a series of standard suspensions, starting from the 0·1 per cent suspension, with 1 per cent sod. citrate as diluent, to give 90, 80, 70 on to 10 per cent suspensions. (12) Prepare a series of tubules from glass tubing free from striæ, of about 4 mm. internal diameter, and of the same calibre and thickness throughout, to contain the series of standard suspensions and also to provide a number of empty tubes for the test suspensions. (13) Seal the standard suspension series of tubes in the blow pipe flame. (14) Use in test by filling in one vol. of the test suspension into one of the empty tubes and adding, if necessary, successive volumes of water until the turbidity of the test suspension corresponds exactly to that of one in the standard series. (15) Calculate<sup>3</sup> the strength of the test suspension.

**Notes.**—<sup>1</sup>Bacteria will grow in the citrate and set up in time a flocculation in the suspension which cannot be broken up. <sup>2</sup>This is best done by placing each of the standard suspension tubes in succession alongside the test suspension tube, in a good light, over clearly printed matter. <sup>3</sup>In the case of organismal suspensions, for which the method is most convenient, a previous original standardization of the organism against the barium sulphate is necessary. This may be done by drying the organism completely, making a homogeneous suspension of a definite weight of the dried organisms and finding its comparative value once and for all in the standard suspension series. This value, which requires to be determined for different organisms separately, is preserved, and calculations are made by simple proportion from this value, e.g., if a suspension of a dried organism at 1 mgm. to the c.c. of menstruum has been shown to be equivalent in turbidity to the standard tube suspension containing 80 per cent of 0·1 per cent barium sulphate, then a suspension of this same organism in the undried condition, which corresponded, say, to the standard tube containing 40 per cent of the 0·1 per cent barium sulphate suspension, would be estimated to contain the equivalent of  $40 \div 80$  mgm. = 0·5 mgm. dried bacterial substance per c.c. of menstruum. A homogeneous suspension of any dried organism is made by soaking a weighed amount of the dried bacterial substance overnight in a small quantity of the menstruum fluid, triturating or shaking to suspend, and making up to the requisite vol. by addition of more diluent. Another method of comparison for organismal suspensions which suggests itself is that of number of

organisms in unit vol. with turbidity as given by the barium sulphate suspension series. The number of organisms contained in a suspension is, however, a variable figure depending on the method of enumeration [S 7-33, S 7-34, S 7-35,] used. This fact has to be kept in mind when adopting number of organisms in unit vol. as the mode of expressing quantity of bacterial substance. Convenient and careful tables have been published showing the correspondence, for a given vol. of fluid, of weight of different organisms in the *dried* state with the degree of turbidity of suspensions, as measured by a standard barium sulphate series of the same organisms in the *undried* state. The following data are taken or extracted by calculation from published tables—(i) 1 mgm. of dried bacterial substance is represented in million of organisms by 3,000 for *Staphylococcus aureus*, 2,800 for *Staphylococcus albus*, 1,553 for *B. typhosus*, 1,419 for *B. coli*, 1,487 *B. paratyphosus A*, 1,915 for *B. dysenteriae* (Flexner), 4,633 for *V. cholerae*, 3,000 for *B. pyocyaneus*, 1,560 for *B. pneumoniae* (Friedländer), 2,204 for *B. proteus*. These figures are valid for a film method of standardization (S 7-351) only. Haemocytometer counts give higher numerical values. (ii) The turbidity of a suspension of 1 mgm. of dried bacterial substance in 1 c.c. of menstruum is in terms of the equivalent dilution of a suspension of 1 per cent. barium sulphate—an 11-fold for *B. typhosus*, *B. paratyphosus A* and *B. B. coli* and *B. enteritidis* (Gartner), 14-fold for *B. pyocyaneus*, 9-fold for *Staphylococcus aureus*, *Staphylococcus albus* and *B. proteus*, 16-fold for *B. pneumoniae* (Friedländer) and *V. cholerae*. (iii) A 0.1 per cent suspension of pure barium sulphate in 1 per cent. *sed. citrate sol* corresponds in turbidity to a suspension in milligrammes of dried substance per cubic centimetre of menstruum of 1.92 for *M. catarrhalis* grown on blood agar, 1.78 for *V. cholerae*, 1.63 for *B. influenzae*, 1.57 for *Gonococcus*, 1.38 for *Pneumococcus*, 1.33 for *B. pyocyaneus* and *Meningococcus* 1.25 for *M. catarrhalis*, 1.14 for *B. dysenteriae* (Shiga and Flexner), 1.10 for *B. typhosus*, *B. paratyphosus A* and *B.* and *B. coli*, 1.06 for *M. melitensis*, 0.94 for *Staphylococcus aureus* and 0.84 for *Staphylococcus albus*, when grown on ordinary agar. The values for turbidities equal to 90, 80, 70 ..... 10 per cent of 0.1 per cent barium sulphate can be calculated by proportion from these values; e.g., if a test suspension of *B. typhosus* from a living culture corresponds to the 60 per cent. suspension, the concentration would be given as 60 per cent of 1.10 mgm. per c.c. = 0.66 mgm. weight of dried bacteria per c.c.

### S 7-13 v. B 8-132.

S 7-14—(1) Dilute the test suspension in a narrow T.T. of 10 to 12 mm. diameter with 0.85 S.S.S. until print 2 mm. in width can be clearly read<sup>1</sup> through it.

Notes.—<sup>1</sup>A turbidity corresponding to 2,000 to 3,000 million *B. typhosus* per c.c.; density that obtained when milk is diluted 200 times with water.

S 7-15.—(1) Use as standard turbidity suspension<sup>1</sup>:—1 per cent mag. sulphate 6; 1 per cent lysol in D. W. 1,000.

Notes.—<sup>1</sup>Equivalent in turbidity to about 27,000 million *B. typhosus* per c.c.

S 7-16 TINTUROMETER.—(1) Use a perfectly clean glass cylindrical measure, with foot free from air bubbles or gross flaws. (2) Let it be of 10 c.c. capacity and of suitable calibre,<sup>2</sup> graduated in 1-10th or 2-10th c.c. (3) Provide the lower half of the cylinder with a covering of black paper or card-board, capable of slipping up and down

the stem. (4) Use a steel rule<sup>2</sup> clearly inscribed with figures as the gauge of turbidity. (5) Add 1 c.c. of 0.1 per cent barium sulphate suspension to the measure. (6) Add 3 c.c. D.W. to give a dilution of 1-4. (7) Mix well. (8) Place the foot-rule on a sheet of white paper in a good light. (9) Stand the cylinder measure over a selected number<sup>3</sup> on the foot-rule. (10) Remove the fluid in quantities of 1-10th or 2-10th c.c. from the cylinder by means of a capillary pipette furnished with a teat, until the selected number just becomes visible<sup>4</sup> when looked down upon through the fluid. (11) Read off the number of c.c. left in the cylinder.<sup>5</sup> (12) Reject the fluid left and clean the cylinder with D. W.<sup>6</sup> (13) Add the test fluid to the cylinder. (14) Proceed as in the case of the standard fluid and find the number of c.c. of test fluid which will give equally minimum visibility of the selected number as the stock standard fluid. (15) Compare this number<sup>7</sup> with that given by the standard barium sulphate suspension, or by a 'normal' fluid.

**Notes.**—<sup>1</sup>A length of cylinder which gives 10 c.c. in about 12 cm. is convenient. <sup>2</sup>A standard print will serve the purpose. The steel rule is convenient because of its permanency and the ease with which it can be cleaned. <sup>3</sup>It is advisable always to use the same number, as the eye becomes accustomed to its appearance. <sup>4</sup>Practice will establish a standard limit for visibility, which, other things being equal, may then be the measure of light absorptive power of the test suspension. It does not matter that the standard may differ slightly in different hands. Each individual establishes his own standard. Until such a standard is so established, or if the conditions of lighting are very variable, it will be necessary to employ a 'normal' or a standard suspension along with the test fluid on each occasion of use. Instead of subtracting fluid until the selected number becomes just visible, the instrument may be used, if desired, in the reverse way—the test fluid may be added to that in the cylinder until the number just disappears from sight. This number, obtained with the standard barium sulphate suspension, is a standard number for the instrument. By its means new instruments may be standardized against the original instrument and thus any determinations with the new instrument made comparable with the old. <sup>5</sup>This is the standard number for the instrument. <sup>6</sup>The cylinder is then ready for further use. The D.W. adhering to the glass should be got rid of with alcohol and ether or by rinsing out the cylinder with a small quantity of the suspension about to be tested. When the instrument is to be put away, wash it out and dry it. After drying, it may be cleaned with abs. alc. followed by ether. Place a wool plug in the mouth of the cylinder to exclude dust. <sup>7</sup>The absolute numbers themselves, without being represented as a fraction of the standard or 'normal,' are sufficient for purposes of inter-comparison, if the same conditions prevail throughout the experiment. Supposing that the original colour of the menstruum and its light absorptive effect has to be considered apart from that of the particles which may be in suspension, the method has to be slightly modified. The effect of the colouration by itself alone has to be measured. The menstruum

as to be freed—as by centrifuging at high speed, or by sedimentation—from its suspended matter. In the case of growth of micro organisms in a fluid medium, the medium may be tested, before growth takes place in it. If  $y^1$  is the figure given for absorption of light by the tintometer for fluid A apart from that due to the presence of suspended particles, and  $y^2$  that similarly for fluid B, while  $y^3$  and  $y^4$  are the figures for these fluids with their suspended particles, then turbidity of fluid A bears the proportion to turbidity of fluid B  $= \frac{y^1}{y^2} \times \frac{y^1 - y^3}{y^2 - y^4}$ ,  $\therefore g.$ , if the tintometer figure for a fluid nutrient medium A was 9 before growth and 1.5 after growth and the figure for a nutrient medium B was 6 before growth and 0.5 after growth, then growth in A  $\div$  growth in B  $= 9 \div 6 \times (9 - 1.5) \div (6 - 0.5) = 2$ . The uses of the instrument are many. It can be used for bacterial suspensions, blood, silt in water, albumin in urine, sugar in urine, etc.

### S 7.2 TINT.

**S 7.21.**—(1) Use one of the various forms of tintometer of which there are very numerous varieties.<sup>1</sup>

**Notes.**—<sup>1</sup>The various makes of hæmoglobinometers are tintometers and can in many cases be adapted for use, if standard solutions for comparison are available, for measuring any type of tint. Thus the hæmoglobinometer of Autenrieth and Koenigsberger, with a wedge capable of being filled with standard tint fluid and an arbitrary scale, is suitable for a variety of purposes.

**S 7.22 TINTOMETER TUBES.**—(1) Prepare a stock standard solution of the requisite tint, with the addition if necessary of a preservative.<sup>2</sup> (2) Prepare a number of tubules 15 in all from the same piece of glass tubing carefully chosen as having the same thickness and calibre throughout and being free from striæ. (3) Prepare from the stock standard sol., by dilution<sup>3</sup> 90, 80, 70, . . . . . 10 per cent solutions. (4) Fill 10 of the tubules prepared, with undiluted stock standard solution and the several dilutions made from it. (5) Seal off the ten tubules.<sup>4</sup> (6) Keep the 5 empty tubules as testing tubules. (7) Carry out the test of comparison of tint in precisely the same way as with the tubules similarly constructed for standardization of turbidity (S 7.12). (8) Calculate<sup>5</sup> the result of comparison for the test solution.

**Notes.**—<sup>1</sup>In the case of hæmoglobin such a stock standard sol. would be given by liberating the hæmoglobin from 1 vol. of the blood of a healthy man with 9 vol. of N 10 hydrochloric acid. It should be remembered that increased altitude of residence results in increase of hæmoglobin content per unit vol. in the healthy man. <sup>2</sup>The N 10 hydrochloric acid serves as a preservative: chloroform, carbolic acid, borax, camphor, thymol, toluol, etc., may also be tried as preservatives. <sup>3</sup>Dilute with N 10 hydrochloric acid. <sup>4</sup>A deposit may form with time consisting probably of the matter which tend to abstract the hæmoglobin from the solution. Simple shaking up of the tubules restores the original standard state. <sup>5</sup>The concentration of the stock standard sol. will, with the degree of dilution of this solution given by the tubule solution which

correspond in tint to that of the test sol., form the basis of the calculation; e.g., if the stock standard sol. is a 1-1000 dilution of hæmoglobin as found in the blood of a healthy man, and a 1-1000 dilution of the test blood corresponds in tint to the sol. contained in the 70 per cent tubule, then the test sol. will be judged to contain per cent of the hæmoglobin of a normal healthy person.

**S 7.23.** (1) Use the tinturometer, for measurement of depth of tint, as described under turbidity (**S 7.16**). (2) Prepare a stock standard sol. of definite tint, with the addition of a preservative if necessary. (3) Fill this sol. from the stock bottle into the tinturometer. (4) Place the foot-rule over a sheet of white paper in a good light. (5) Stand the cylinder measure over a selected number on the foot-rule. (6) Remove the fluid in quantities of 1-10th or 2-10th c.c. from the cylinder by means of a capillary pipette furnished with a teat, until the selected number just becomes visible when looked down upon through the fluid. (7) Read off the number of c.c. left in the cylinder. (8) Reject the fluid left and clean the cylinder with D.W. (9) Add some test fluid to the cylinder to rinse it out. (10) Add the test fluid to the cylinder and find the number of c.c. test fluid which will give equally minimum visibility of the selected number as the stock standard sol. (11) Compare this number with that given by a 'normal' fluid or by the stock standard sol.

### **S 7.3 BACTERIAL ANTIGEN.**

#### **S 7.31 BY WEIGHT**

**S 7.311 DRIED**—(1) Desiccate the antigen to constant weight. (2) Weigh out the quantity necessary to make a suspension of definite strength. (3) Make into a suspension with the requisite amount of 0.85 S.S.S. or other menstruum.

**S 7.312 UNDRIED.**<sup>1</sup>—(1) Remove the organismal growth from a 24-hours culture on an agar slope. (2) Place it in a weighed watch glass. (3) Weigh the watch glass and contents rapidly on a chemical balance. (4) Calculate the weight of the moist organismal growth. (5) Add a definite quantity<sup>2</sup> of 0.5 per cent carbolyzed 0.85 S.S.S. or other menstruum. (6) Work up the growth in the salt sol. by means of a glass rod to give a uniform suspension. (7) Dilute further with 0.5 per cent carbolyzed 0.85 S.S.S. or other menstruum to give the concentration<sup>3</sup> desired.

**Notes.**—<sup>1</sup>The objection to the standardization of moist organismal growth by weight is that it varies with the amount of moisture taken up in culture. <sup>2</sup>E.g., 1 c.c. <sup>3</sup>E.g., 1 mgm. per c.c.

**S 7-32 BY TURBIDITY.**—(1) Use methods S 7-12, S 7-13, S 7-16.

**S 7-33 BY HÆMOCYTOMETER COUNT.**

**S 7-331.**—(1) Prepare a suspension of washed erythrocytes in the same fluid as the test organism is suspended in. (2) Count the number of erythrocytes contained in unit vol. of the erythrocyte suspension by hæmocytometer. (3) Mix 9 vol. test bacterial suspension with 1 vol. 1 per cent methylene blue. (4) Place the stain and test bacterial suspension in a T.T. in a water bath at 45°C. (5) Raise the temperature of the water bath to 60°C. (6) Keep at this level 15 min.<sup>2</sup> (7) Mix a suitable number<sup>3</sup> of vol. of stained bacterial suspension with 1 vol. erythrocyte suspension. (8) Place a droplet of the mixture on a slide. (9) Cover immediately with a cover glass and lute with vaselin. (10) Make 2 or 3 such preparations. (11) Examine under a 1-12th in. immersion lens. (12) Count field by field<sup>4</sup> the number of stained bacteria<sup>5</sup> and erythrocytes present. (13) Calculate<sup>6</sup> from the results the number of bacteria in 1 c.c. of bacterial suspension.

**Notes.**—<sup>1</sup>Usually 0.85 S.S.S. or nutrient bouillon. <sup>2</sup>The temperature must be kept constant and must not be exceeded, else there is a tendency for the stain to form large blocks of deposit. <sup>3</sup>A trial may be made of 10 vol. <sup>4</sup>About 50 fields should suffice. <sup>5</sup>These will be found to be in different planes and require up and down focussing for enumeration. <sup>6</sup>Nine-tenths the number of bacteria contained in 1 c.c. test bacterial suspension = (No. of erythrocytes in 1 c.c. erythrocyte suspension ÷ No. of erythrocytes counted) × (No. of bacteria counted ÷ No. of vol. of bacterial suspension used). The figure 9-10 in this calculation is due to the fact that the test bacterial suspension is diluted with 1-10th of its vol. of stain and a correction is necessary for this.

**S 7-332.**—(1) Use as diluting fluid for the test bacterial suspension 1 part sat. thionin blue in abs. alc. to 40 parts 1 per cent phenol. (2) Filter before use. (3) Use a counting chamber 0.1 or better 0.02 mm. deep. (4) Allow time for settlement. (5) Count a sufficiency of squares, paying special attention to the organisms adherent to the under surface of the cover glass.

**Notes.**—<sup>1</sup>If the thionin in the stain is too concentrated an abnormal number of stained bacteria adhere to the under surface of the coverslip. Agglutination of bacteria also occurs. Other staining and diluting solutions which may be used are:—(i) 0.05 per cent dithia in 1 per cent formalin, (ii) hydrochloric acid 2; 1-500 mercuric chloride 100; 1 per cent. fuchsin in sufficient quantity to give a deep cherry red colour. The mercuric chloride form an albuminate on the surface of the bacteria, which promotes rapid sedimentation. (iii) formalin 2; alc. gentian violet 5; 1 per cent sod. chloride 100. (iv) carbol fuchsin 0.25; 5 per cent phenol 100.

**S 7·34 BY COLONY COUNT.**

**S 7·341.**—(1) Use sterile capillary pipettes<sup>1</sup> with slightly upturned ends, calibrated to 100 c.mm. capacity. (2) Make suitable dilutions 1-100, 1-10,000, etc., by adding 100 c.mm. from the test suspension to a T.T. containing 10 c.c. 0·85 S.S.S. or sterile bouillon, and from that carrying over the same amount to a second T.T. containing 10 c.c. of diluent, and so on. (3) Shake to mix. (4) Take up 100 c.mm. from the 1-100 dilution of the test suspension. (5) Place 3 successive portions of this quantity on 3 dry agar T.T. slopes, rubbing the quantum ejected well in over each slope. (6) Rest each T.T. without its wool plug on glass tubing or other support in as nearly horizontal a position as possible. (7) Allow to dry in this position. (8) Sterilize the mouths of the T.T. and the corresponding wool plugs. (9) Replace the wool plugs in the T.T. (10) Incubate. (11) Proceed in similar fashion for the 1-10,000 and other dilutions. (12) Count the colonies which develop. (13) Calculate from the number of colonies the number of organisms contained in 1 c.c. of the test suspension.

**Notes.**—<sup>1</sup>Thirty or more of these calibrated pipettes can be made in an hour. <sup>2</sup>Upturned to avoid scratching the surface of the agar slope. <sup>3</sup>Bouillon is a better diluent than salt sol. <sup>4</sup>Strictly speaking, should be 9·9 c.c. instead of 10 c.c. <sup>5</sup>If fresh sterile capillary pipettes are not available, the one pipette may be used for all operations by sterilizing at each separate step in boiling water. The boiling water is simply aspirated into and ejected several times from the pipette. <sup>6</sup>It is highly important to have the agar slope thoroughly dry. This may be done by placing the T.T. in the incubator on the evening previous to test in an inverted position. Water of condensation is taken up by the wool plug. <sup>7</sup>Thus if in the 3 T.T. used on which to plant out 100 c.mm. there developed 8, 12, and 10 colonies, respectively, making a total of 30 colonies, and the dilution used was 1-10,000, the calculation would be  $30 \times 10 \times 10,000 = 3,000,000$  organisms per c.c. in the test suspension.

**S 7·342.**—(1) Use 10 c.mm. calibrated capillary pipettes (**S 9·21**). (2) Sterilize and fit with rubber teats. (3) Have in readiness 3 T.T. containing, respectively, 10 c.c., 1 c.c. and 10 c.c. sterile bouillon in each. (4) Make No. 1 dilution of the test suspension by transferring 10 c.mm. to No. 1 T.T. containing 10 c.c. bouillon = 1-1,000 dilution. (5) Wash out the pipette well with the bouillon. (6) Shake to mix. (7) Make No. 2 dilution by transferring with a fresh pipette 10 c.mm. from No. 1 dilution to No. 2 T.T. containing 1 c.c. bouillon = 1-100,000 dilution. (8) Wash out the pipette well with the bouillon. (9) Shake to mix. (10) Make No. 3 dilution in the same way by transferring 10 c.mm. from No. 1 dilution to No. 3 T.T. containing 10 c.c. bouillon = 1-1,000,000 dilution.<sup>1</sup> (11) Use a sterile 100 c.mm. calibrated

capillary pipette (S 9·21) fitted with a rubber teat. (12) Mark off a  $4\frac{1}{2}$  in. Petri dish containing nutrient agar into 4 equal sectors by means of lines drawn with a grease pencil on the bottom of the dish. (13) Plant out by droplets<sup>2</sup>, or by a succession of closely parallel lines, 100 c.mm. of No. 3 dilution on each of 2 sectors of the Petri plate and 100 c.mm. of No. 2 dilution on each of the remaining two sectors. (14) Incubate the plate in the inverted position 24 hours. (15) Count the colonies which have developed. (16) Calculate<sup>3</sup> the number<sup>4</sup> of organisms in the original suspension.

**Notes.**—<sup>1</sup>There are many other ways of making the necessary dilution of the test suspension. If it is not convenient to work with capillary pipettes, dilutions may be made by 10-fold steps by transferring 1 c.c. of suspension from each of a succession of T.T. containing 9 c.c. bouillon to the other. Instead of bouillon 0·85 S.S.S. may be used as the diluent. <sup>2</sup>These droplets are very minute and represent roughly about 0·5 c.mm. each. With the dilution used they may be expected to contain not more than 1 organism<sup>5</sup> in each droplet. <sup>3</sup>If in 2 sectors taken together sown from the 1:1,000,000 dilution 80 colonies develop, then the original test suspension will contain  $\frac{80}{2} \times 1,000,000 \times 10 = 400,000,000$  organisms per c.c. <sup>4</sup>This method gives strictly the number of *living* organisms only contained in the test suspension. Clumps of organisms, too, will develop as a single colony.

### S 7·35 BY FILM PREPARATION.

**S 7·351.**—(1) Take a simple capillary pipette fitted with a teat and make a mark upon the stem about 1 in. from the tip. (2) Prick the finger and press out a drop of blood. (3) Draw up into the pipette first one vol. of blood and then, with an air column separation, 1 or more vol. of bacterial suspension according to the concentration of the latter.<sup>1</sup> (4) Follow on with 2 vol. of D.W.<sup>2</sup> (5) Expel the D.W. and bacterial suspension first, and mix. (6) Add the vol. of blood and mix the whole well by aspirating into and expelling from the pipette. (7) Make from the mixture 2 or more films. (8) Fix 2 min. in sat. mercury bi-chloride. (9) Wash well. (10) Stain 4 min. with filtered carbol thionin. (11) Examine the films to see whether they are satisfactory.<sup>3</sup> (12) Make a differential count with an oil immersion lens and high eyepiece<sup>4</sup> of the number of bacteria and erythrocytes in each field.<sup>5</sup> (13) Calculate<sup>7</sup> the number of bacteria in 1 c.c. of test suspension.

**Notes.**—<sup>1</sup>Use 2 or more vol. if it appears from an examination that the suspension will give very much less than an equal number of bacteria for counting. <sup>2</sup>Or 1·5 per cent citrated 0·85 S.S.S. <sup>3</sup>So that there may be no danger of the erythrocytes being hemolysed. <sup>4</sup>The erythrocytes and bacteria should preferably be in approximately equal number on the film and there should not be present enough

of bacteria or erythrocytes. <sup>2</sup>The high eyepiece restricts the field of view and so reduces the number of elements to be enumerated. An Ehrlich eyepiece or a paper diaphragm dropped on to the diaphragm of the eyepiece will serve the purpose also. <sup>6</sup>Pass at random from field to field, traversing every region of the slide. <sup>7</sup>Assume that the blood contains 5,000 million erythrocytes per c. c. then :—  
 No. of bacteria in 1 c.c. of the test suspension = (No. of bacteria counted ÷ No. of erythrocytes counted) × (No. of vol. of test bacterial suspension ÷ No. of vol. of blood used) × 5,000,000,000.

**S 7·352**—(1) Use as standard a previously counted suspension<sup>1</sup> of organisms of convenient strength<sup>1</sup> for comparison with the test suspension, consisting of Gram-negative<sup>1</sup> organisms when the test organism is Gram-positive and of Gram-positive<sup>1</sup> organisms when the test organism is Gram-negative. (2) Dilute the test suspension to approximately the same opacity as the standard suspension and note the degree of dilution required. (3) Mix equal vol. of standard suspension and diluted test suspension. (4) Make films on slides from the mixture. (5) Fix the films by heat. (6) Stain the films by Gram's method and counterstain with 2 per cent neutral red. (7) Make a differential count with an oil immersion lens and high eyepiece of the number of test suspension bacteria and the number of standard suspension bacteria in each field. (8) Calculate<sup>5</sup> the number of bacteria in 1 c.c. of test suspension.

**Notes.**—<sup>1</sup>This suspension, with the addition of some 2 drops of formalin, will keep well and serve for a long time as a standard. <sup>2</sup>*E.g.*, 1,500 to 2,000 million p.r.c.c. <sup>3</sup>*E.g.*, *B. coli*. <sup>4</sup>*E.g.*, *Staphylococcus aureus*. <sup>5</sup>No. of bacteria in 1 c.c. test suspension = (No. of test suspension organisms counted ÷ No. of standard suspension organisms counted) × No. of organisms in 1 c.c. of standard suspension × degree of dilution of test suspension.

### **S 7·36 BY AREA OF GROWTH.**

**S 7·361**—(1) Make a suspension of seed culture in 0·85 S.S.S. (2) Dilute it to contain, say, 1,000 million organisms per c.c. or estimate the bacterial content in terms of weight and dilute to convenient strength. (3) Sow 10 c.mm. of the diluted suspension over the whole of a definite area<sup>1</sup> of an agar slope. (4) Incubate 24 hours. (5) Add as many c.c. of 0·85 S.S.S. to the T.T. as there are square centimetres in the area selected for growth. (6) Make a suspension<sup>2</sup> of the growth in the added fluid.

**Notes.**—<sup>1</sup>The breadth of the agar slope at the middle is estimated by means of a centimetre rule, and 2 parallel lines drawn across the tube at a convenient distance from one another indicate the upper and lower limits of sowing. <sup>2</sup>Each c.c. of this suspension will represent the yield from a square centimetre of medium.

**S 7362.**—(1) Sow completely the growth area of a slope agar tube. (2) Incubate 24 hours. (3) Estimate the area of growth in square centimetres by measuring its length and its breadth and multiplying the two figures together. (4) Add as many c.c. of suspending fluid to the tube as there are square centimetres of growth. (5) Make a suspension of the growth in the fluid.<sup>1</sup>

**Notes.**—<sup>1</sup>Each c.c. of the suspension will represent the yield from a square centimetre of medium.

#### **S 737 BY PLATINUM LOOP.**

**S 7371.**—(1) Pick up from a growth on agar a given number of standard loopfuls<sup>1</sup> and suspend in a measured quantity of 0.85 S.S.S.

**Notes.**—<sup>1</sup>A platinum loop of 4 mm. internal diameter holds approximately 2 mgm. or roughly 2,500,000,000 organisms.

#### **S 738 BY MINIMAL LETHAL DOSE.<sup>1</sup>**

**Notes.**—<sup>1</sup>See also B 8.9. B 9.2

**S 7381<sup>1</sup>.**—(1) Use 50 G. P. of weight 350-400 gm. (2) Inject subcutaneously 0.5 mgm. of the 13-day living growth of *B. tuberculosis* in bouillon, contained in a vol. of 0.5 c.c. 0.85 S.S.S. (3) Weigh the animals to determine, from the loss of weight, when they have become infected and are ready for test.<sup>2</sup> (4) Dilute the test tuberculin 10-fold. (5) Inject a series of the prepared G. P. subcutaneously with, say, 0.5, 0.75, 1, 1.5, 2 and 3 c.c. of the diluted test tuberculin. (6) Take the smallest dose of tuberculin which kills<sup>3</sup> a prepared G.P. within 24 hours as the titre<sup>4</sup> of the test tuberculin.

**Notes.**—<sup>1</sup>Animal test. The example chosen in illustration of the M.L.D. method is that of tuberculin. <sup>2</sup>It is well to make a preliminary trial on a few of the G.P. This trial would take place usually in the third week after inoculation of living *B. tuberculosis*. A standard tuberculin may be used for the preliminary trial in doses, subcutaneously, of 0.3, 0.4, and 0.5 c.c. If guinea-pigs in this preliminary trial die in 24 hours, the main trial may be proceeded with. If none die within 24 hours, it is advisable to wait a little longer before making the test. <sup>3</sup>A P.M. must be made to determine the actuality of death from tuberculosis. <sup>4</sup>A number of the 50 G.P. is usually set apart on which to determine in the same way the M.L.D. of a standard tuberculin. The test tuberculin can thus be compared directly as regards titre with an accepted standard.

**S 7382** v. also **S 74.**

#### **S 739 BY AGGLUTINABILITY.**

**S 7391 SUSPENSION.**—(1) Subculture the organism to be used in test daily in bouillon two weeks or longer. (2) Use ordinary veal peptone bouillon, (3) neutralized<sup>2</sup> to phenolphthalein, in partially

filled flasks. (3) Sterilize (**S 9·6**) not more than 15 min. at 115C. (4) Test for sterility by incubation 48 hours. (5) Sow the flasks from a 20 to 24 hours culture of the antigen organism. (6) Incubate the flasks 24 hours. (7) Shake well. (8) Add to each flask 0·1 per cent formalin. (9) Shake and place in the ice chest at 2C. (10) Shake at intervals for 5 d. always replacing immediately in the ice chest. (11) Place in cold storage for 2 m. to become stabilized.<sup>1</sup>

**Notes.**—<sup>1</sup>This subcultivation has the effect of increasing its agglutinability and diminishing any tendency to spontaneous agglutination. <sup>2</sup>The bouillon is titrated against phenolphthalein and  $\frac{1}{3}$  rds of that amount of sod. hydroxide which would render it neutral to phenolphthalein added to it. <sup>3</sup>Changes, though at first rapid and not inconsiderable in the case of certain organisms, become, after a few months' storage in the dark at low temperature, so slight and slow that both suspension and serum may, for practical purposes, be regarded as stable for at least one or even two years.

**S 7·392 STANDARDIZATION.**—(1) Make the formalinized culture (**S 7·391**) by dilution with 0·1 per cent formalinized 0·85 S.S.S. approximately equal in opacity to that of the standard agglutinable culture. (2) Make a dilution of the 'standard agglutinin serum' of such a strength that 1 c.c. contains 4 to 8 standard agglutinating units. (3) Make a series of dilutions 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20 in 20 of the diluted standard serum. (4) Set up 2 racks of 12 tubules each. (5) Add to the tubules of No. 1 rack 2 vol., in order, of each of the dilutions of the diluted standard serum. (6) Add the same amount and same series of dilutions to each of the tubules of rack No. 2. (7) Add to each tubule in No. 1 rack 3 vol. of standard agglutinable culture and to each tubule in No. 2 rack 3 vol. of the test formalinized culture. (8) Keep the tubules 2 hours (dysentery  $4\frac{1}{2}$  hours) in a water bath at 50 to 55C. (9) Remove and leave 15 to 20 min. at R.T. (10) Read by selecting the tube in the standard agglutinable culture series which exhibits standard agglutination<sup>1</sup> and ascertain which tube in the other series shows the same degree of agglutination. (11) Repeat the comparison and make more exact by selecting in succession from the first series one or two other tubes which exhibit less than standard agglutination and similarly matching them in the second series. (12) Compare the agglutinabilities of the 2 suspensions and give the test formalinized suspension the reduction factor which makes it equivalent to standard agglutinable culture.

**Notes.**—<sup>1</sup>Standard agglutinating serum is not absolutely necessary. Any serum diluted so as to bring out standard agglutination within its range of use will serve as the means by which the test formalinized suspension is compared with the standard

agglutinable culture. <sup>2</sup>By artificial light, against a dark background. <sup>3</sup>The highest dilution in which marked agglutination without sedimentation can be detected by the naked eye. <sup>4</sup>E.g., if the standard agglutinable culture is found to be, say, twice as agglutinable as the killed culture, the factor to be given to the latter to make it standard will be the factor supplied with the standard agglutinable culture divided by 2. If, however, the standard agglutinable culture is found to be, say, half as agglutinable as the killed culture, the factor to be given to the latter to make it standard will be the factor supplied with the standard agglutinable culture divided by  $\frac{1}{2}$ . The number of agglutinin units in a test serum is obtained by dividing the dilution which gives standard agglutination by the factor attaching to the standard agglutinable culture which is used in the test.

**S 7393 STANDARDIZATION.**—(1) Make a trial determination of the titre<sup>1</sup> of the high titre serum<sup>2</sup> used, to the test antigen and the standard agglutinable culture. (2) Dilute<sup>3</sup> the serum to contain twice the amount of serum contained in the titre dilution. (3) Make dilutions<sup>4</sup> again in watch glasses of this dilution of 1, 1-125, 1-25, 1-375, 1-5, 1-625, 1-75, 1-875 and 2-fold degree. (4) Use 1 vol. of this series of dilutions in mixture with 1 vol.<sup>5</sup> of test and of standard antigen suspension to determine the titre of the serum to each. (5) Compare the results in any way convenient to fix the agglutinability of the test antigen in terms of standard agglutinable culture. (6) Use the test antigen if desired as the standard antigen with the fixed factor of conversion.

**Notes.**—<sup>1</sup>Any of the various expressions of titre may be taken for the purpose. <sup>2</sup>Any high titre serum may be used. <sup>3</sup>This standard suspension is one which is arbitrarily chosen in the first instance, but which once chosen becomes the standard against which all laboratory suspensions which may subsequently be made up are tested for their agglutinability. The standard suspension is replaced, when it becomes used up by another suspension, which has been standardized against it. <sup>4</sup>That is, a 2-fold concentration of the titre dilution. If the titre dilution is 1-200, this dilution will be 1-100. <sup>5</sup>For example, a 1-125-fold dilution of a serum is calculated as follows:  $1-125-1=0.125$ . An addition of 0.125 vol. of diluent to 1 vol. of serum will give the required result. This is equivalent to 8 vol. of serum plus 1 vol. of diluent. <sup>6</sup>Where vol. is used in the description this term may be replaced by the word "drops." A drop or a number of drops is a more easily, more rapidly measured unit vol. than that obtained by drawing up fluid in volumes to a fiducial mark on a capillary stem. The drop may be of any size if used consistently throughout the operations. A capillary pipette furnished with a teat makes an excellent dropper. It requires to be well washed out in transfers from serum to salt solution or suspension, or from more concentrated serum to less concentrated. The final dilutions of the serum dilution used in the series given are 1-2, 2-25, 2-5, 2-75, 3, 3-25, 3-5, 3-75 and 4. <sup>7</sup>As it is a comparison of agglutinability, when a being made, any point in the one series which gives an identical result with any point in the second series may be taken for the determination of equivalence, whether an end point or not.

**S 7·4 TOXIN<sup>1</sup>.**

**Notes.**—<sup>1</sup>The standardization of diphtheria and tetanus toxins and their antitoxins is a complicated procedure and the description here given is merely an indication of the nature of that procedure. See **B. 8·91** and **B 8·92** for more detailed description.

**S 7·41. DIPHTHERIA TOXIN.**

**S 7·411.**—(1) Obtain a standard antitoxic serum. (2) Mix one unit of this standard antitoxic serum with varying quantities of the test toxin. (3) Inject the mixture subcutaneously into a series of 250 grm. guinea pigs. (4) Determine from this series of G. P. the 'Lo dose' and the 'L + dose'.<sup>3</sup>

**Notes.**—<sup>1</sup>The standard antitoxin unit is that quantity of antitoxin which will neutralize 100 times the minimum lethal dose of toxin for a 250-grm. G.P. This pure fresh toxin has to be arbitrarily selected in the first instance. It is used to standardize an antitoxic serum, which is then carefully preserved in the desiccated condition as the standard material for testing toxin and antitoxin. By 'pure toxin' is meant a substance containing only toxin and not toxone, toxid, or other substance capable of combining with antitoxin. <sup>2</sup>The Lo dose is that quantity of toxin which is just capable of neutralizing one standard antitoxin unit, as shown by survival of the G.P. and by a detailed P.M. examination to show absence of reaction. <sup>3</sup>The L + dose is the smallest quantity of toxin that will neutralize in mixture one standard antitoxin unit, and leave sufficient toxin unneutralized to kill in 4 d. a 250-grm. G.P. which has received the toxin antitoxin mixture subcutaneously. See also **B 8·9138**.

**S 7·24 TETANUS TOXIN.**—(1) Inject subcutaneously increasing amount of precipitated<sup>1</sup> desiccated toxin into a series of 350-grm. G.P. (2) Determine the minimum lethal dose from the deaths in the series of G.P. used. (3) Preserve the desiccated toxin carefully for use as a standard for testing toxin and antitoxin.<sup>1</sup>

**Notes.**—<sup>1</sup>Precipitation with ammon. sulphate. <sup>2</sup>In the case of *B. tetani*, it is the dried toxin which is preserved as the standard measure of potency of antitoxic sera, whereas in the case of *B. diphtherie* it is a dried antitoxic serum which serves as measure. <sup>3</sup>V. **B 8·92**.

**S 7·43 SNAKE VENOM.**

**S 7·431.**—(1) Inject increasing amount of dried cobra venom dissolved in sterile D.W. into the ear vein of a series of rabbits. (2) Determine the smallest amount which kills in 15 to 20 min. (3) Use the dried venom, thus standardized for the standardization of antivenomous serum.

**Notes.**—<sup>1</sup>To standardize :—(i) inject a series of 2-kilogramme rabbits intravenously with 0·5, 1, 2, 3 c.c., etc., test anti-serum. (ii) follow this injection 15 min. later with one in the other ear vein of the determined minimum lethal dose of standard venom. This minimum lethal dose should be re-determined from time to time. If 1 c.c.

is the minimum quantity of serum which serves to save a 2 kilogramme rabbit from the lethal effect of the minimum lethal dose of standard venom, the serum is said to contain 2 000 units per c.c. and so in proportion to the amount of serum required, *V.* also **B 9.2.**

**S 7.432.**—(1) Use cobra venom which has been desiccated *in vacuo* over sulphuric acid, or calc. chloride, and preserved in the desiccated state in a sealed tube in a cool, dark place. (2) Use as a standard 1 mgm. of desiccated venom, contained in 1 c.c. 0.85 S.S.S. (3) Standardize<sup>1</sup> test antivenins against this dose.

**Notes.**—<sup>1</sup>The standardization of the antivenin may take the very simple form of a pronouncement on the capability of 1 c.c. of serum to preserve the life of a rabbit, for 24 hr. at least, against a dose of 1 mgm. of venom per kilogram. weight of rabbit, when the mixture of the two is injected subcutaneously. *V.* also **B 9.2.**

**S 7.5 POLLEN. V. A 3.321. B 8.53.**

**S 7.6 WASSERMANN TEST ANTIGEN. V. WASSERMANN TEST (B 9.8. B 10.9).**

**S 7.7 STANDARD SOLUTIONS.**

**Note.**—<sup>1</sup>Comprising those mentioned in this work, which are not actually given along with the detail of the technique in the test in which used.

**S 7.71 NORMAL ALKALI.**

**S 7.711**—(1) Place a small quantity of the purest obtainable sod. bicarbonate in a platinum<sup>1</sup> capsule. (2) Heat with a moderate flame<sup>2</sup> for several hours. (3) Place in desiccator to cool over sulphuric acid. (4) Weigh the capsule and its contents. (5) Heat again, cool and re-weigh. (6) Continue the procedure until 2 successive weighings show no significant difference in weight. (7) Remove a quantity of the anhydrous sod. carbonate from the capsule and dissolve with gentle heat in a definite but small quantity of D.W. (8) Weigh the capsule to determine the exact weight of sod. carbonate removed. (9) Make up the solution obtained, by addition of a further amount of D.W., to normal strength.<sup>3</sup>

**Notes.**—<sup>1</sup>Sod. carbonate at a high temperature will attack the glaze of a porcelain capsule and hence such a capsule cannot be used. <sup>2</sup>Too high a temperature would result in some conversion to oxide. <sup>3</sup>5.3 grm. per litre.

**S 7.712.**—(1) Weigh a platinum capsule. (2) Place in it a quantity of anhydrous sod. carbonate 'pure for analysis.' (3) Heat with a moderate flame. (4) Place in a desiccator to cool over sulphuric acid. (5) Place the capsule with contents on a balance. (6) Remove quickly that amount of sod. carbonate which will leave in the capsule exactly 5.3 grm. (7) Dissolve this quantity left in the capsule in D.W. with gentle

heat in a tared litre flask. (8) Allow to cool. (9) Make up to one litre with D.W.=N-1 alkali.

**S 7·713.**—(1) Place 1,100 c.c. D.W. in a clean glass stoppered bottle. (2) Drop in quickly 4 complete sticks of caustic soda from a newly opened bottle. (3) Replace the stopper and allow the sticks to dissolve with occasional gentle shaking. (4) Fill a graduated burette with some of the solution. (5) Place 100 c.c. N-10 hydrochloric acid (**S 7·723**) in a beaker and add 2 drops of phenolphthalein indicator sol. (**S 7·772**). (6) Run in the caustic soda sol. slowly from the burette until a single drop changes the colourless sol. to a well-defined permanent but faint pink. (7) Note the amount of caustic soda sol. used.<sup>1</sup> (8) Calculate the amount of D.W. to be added to the caustic soda sol. to make it N-1 alkali.

**Notes.**—<sup>1</sup>It should be less than 10 c.c. If it is more than 10 c.c., then another half stick should be added to the litre of sol. which should then be re-titrated <sup>2</sup>*E.g.*, if the amount of caustic soda sol. used to neutralize the 100 c.c. N-10 hydrochloric acid be, say, 8·5 c.c., then each 8·5 c.c. must be made up to 10 c.c. to give N-1 alkali.

#### **S 7·72 NORMAL ACID.**

**S 7·721.**—(1) Powder some well formed crystals of pure oxalic acid. (2) Dry between filter paper to remove extraneous moisture. (3) Weigh out exactly 63·024 gm. and place in a litre flask. (4) Wash out the container of the weighed quantity of oxalic acid with D.W. into the flask. (5) Heat gently to dissolve. (6) Allow to cool. (7) Bring up to the litre vol. exactly. (8) Titrate against normal alkali and use the figure obtained as factor of conversion.

**Notes.**—<sup>1</sup>Cannot be made into a normal sol. by direct weighing alone owing to uncertain hydration.

**S 7·722.** (1) Add 27 c.c. strong sulphuric acid gradually to 900 c.c. D.W. (2) Allow to cool. (3) Fill a 50 c.c. burette with normal alkali (**S 721**). (4) Place 20 c.c. of the acid sol. in a porcelain evaporating dish. (5) Add 2 drops of phenolphthalein indicator sol. (**S 7·772**). (6) Run in the normal alkali sol. slowly and under control from the burette until a single drop changes the colourless sol. to a well defined permanent pink. (7) Note the amount of normal alkali sol. used. (8) Calculate the amount of dilution<sup>2</sup> required to make the acid sol. a normal one.

**Notes.**—<sup>1</sup>If normal sod. carbonate be used, the indicator sol. should be methyl. orange. (**S 7·773**) <sup>2</sup>*E.g.*, if 22·5 c.c. of normal alkali have been used, then each 20 c.c. of the acid sol. will require to be made up to 22·5 c.c. to become a normal sol.

**S 7-723 N-10 HYDROCHLORIC ACID.**<sup>1</sup>—(1) Weigh out 1.699 gm. pure<sup>2</sup> silver nitrate. (2) Dissolve in a small quantity of D.W. and make up accurately to 100 c.c. at 150° N-10 sol. (3) Keep in a dark place. (4) Place 6.5 c.c. colourless strong hydrochloric acid in a stoppered litre flask. (5) Fill up to the litre mark with D.W. (6) Invert several times until mixing is complete. (7) Add D.W. beyond the litre mark almost up to the stopper. (8) Invert several times to mix thoroughly. (9) Transfer exactly 10 c.c. of this acid sol. to a porcelain evaporating dish. (10) Add 2 or 3 drops of pot. chromate sol. **S 7-774**. (11) Run in the silver nitrate sol. slowly, stirring constantly with a glass rod. (12) Continue until a single drop produces a pinkish red colour. (13) Calculate<sup>3</sup> the equivalence of the acid and N-10 silver nitrate solutions. (14) Make<sup>1</sup> up the acid sol. to decinormal.

**Notes.**—<sup>1</sup>Normal hydrochloric acid may also be made up and standardized described for sulphuric acid (**S 7-722**) using 1.3 c.c. strong hydrochloric acid to 10 c.c. D.W. as the crude sol. <sup>2</sup>Purify, if necessary, by crystallization and recrystallization from D.W. <sup>3</sup>Deduct from the amount of silver nitrate sol. used 0.05 c.c. for the last drop causing colouration. The remainder will be equivalent to 10 c.c. of the acid sol. <sup>4</sup>If 11.7 c.c. N-10 silver nitrate sol. are equivalent to 10 c.c. acid sol., then each 10 c.c. of the acid sol. will require to be made up to 11.7 c.c. to become a decinormal sol. The decinormal sol. so made up should be titrated against the N-10 silver nitrate sol. to confirm their equivalence.

**S 7.73 1-15 MOLECULAR DI-HYDROGEN POT. PHOSPHATE.**<sup>1</sup>—(1) Prepare pure di-hydrogen pot. phosphate by recrystallizing the salt 3 times from D.W. (2) Dry between filter paper and then in a water oven at 100°C. (3) Weigh out 9.078 gm. and dissolve in about 500 c.c. D.W. contained in a tared litre flask. (4) Add 45.5 c.c. phenol sulphone phthalein indicator sol. (**S 7.775**). (5) Make the whole up to 1,000 c.c. with D.W.

**Notes.**—<sup>1</sup>Acid pot. phosphate,  $\text{KH}_2\text{PO}_4$ . Used for colorimetric determination of hydrogen ion concentration.

**S 7.74 N-5 DI-HYDROGEN POT. PHOSPHATE.**<sup>1</sup>—(1) Weigh out 13.616 gm. di-hydrogen pot. phosphate. (2) Dissolve in about 300 c.c. boiled D.W. (3) Add 90.6 c.c. phenol sulphone phthalein indicator sol. (**S 7.7'5**). (4) Make up the mixture to 500 c.c.

**Notes.**—<sup>1</sup> $\text{KH}_2\text{PO}_4$ . Used for colorimetric determination of hydrogen ion concentration.

**S 7.75 1-15 MOLECULAR DI-HYDRATE OF SOD. PHOSPHATE.**—(1) Place a quantity of crystalline sod. phosphate B. P.

which should be of the purest quality, in a flat dish, in a layer not more than 1.5 cm. deep. (2) Cover with paper to protect from dust. (3) Allow to stand two weeks at R.T. in a dry atmosphere. (4) Turn over the salt with a spatula every second day and crush any of the larger lumps.<sup>2</sup> (5) Weigh out 11.876 gm. of the di-hydrate of sod. phosphate so prepared and dissolve in about 500 c.c. D.W. in a tared litre flask. (6) Add 45.5 c.c. phenol sulphone phthalein indicator sol.<sup>3</sup> (S 7.775). (7) Make up the vol. to 1000 c.c.

**Notes.**—<sup>1</sup>At the end of two weeks the salt is transformed into a snow white granular substance having the composition  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ . <sup>2</sup>A check may be made during the last few days by weighing out about 100 gm. with an accuracy of 0.1 gm. and weighing again in the course of 2 d. The two weights should agree to 0.1 gm. It is advisable to have the water content of the salt checked by analysis. This may be done by determining the loss in weight when the salt is dried to constant weight at 98°C in a water oven. The loss should equal 2.02 per cent of the weight of the di-hydrate. A 1 per cent sol. should give a red colour with a few drops of phenol phthalein indicator sol. (S 7.772). <sup>3</sup>The indicator sol. is required to give a colorimetric series for pH measurement (S 7.61).

#### S 7.76 STANDARD HYDROGEN ION MIXTURES.

**S 7.761.**—(1) Have in readiness:—50 c.c. standard burettes graduated in 1-10ths., standard 100 c.c. flasks, 'cordite tubes' for the standard mixtures. (2) Clean the cordite tubes thoroughly by boiling with D.W. (3) Rinse out several times. (4) Dry in the water oven. (5) Draw out in the blow pipe flame to give a length of tube about 8 cm., and to leave a constriction about 3 mm. in diameter through which the tubes can be filled. (6) Make a small capillary funnel from a T.T. by drawing out a neck less than 3 mm. in diameter and about 15 cm. long. (7) Measure the requisite amount of 1-15 molecular di-hydrate of sod. phosphate sol. (S 7.75) containing phenol red indicator into carefully cleaned, tared, 100 c.c. flasks for the given hydrogen ion mixtures and make up the volumes exactly to 100 c.c. with 1-15 molecular dihydrogen pot. phosphate sol. (S 7.73). (8) Fill into the 'cordite' tubes the mixtures so obtained by means of the small capillary funnel. (9) Place a few crystals of thymol in the tubes to prevent any bacterial growth. (10) Seal each of the tubes in the blow pipe flame at the constriction. (11) Number them consecutively and inscribe upon each of them its pH. value. (12) Keep in the dark when not in use.

**Notes.**—<sup>1</sup>Cordite tubes are specially selected tubes of hard white glass which have a uniform thickness of wall and external diameter. They are used in certain colour reactions in the routine testing of cordite. Any T.T. of good quality may be used,

provided that a careful selection be made so that they are all of the same measurements. Use the following amounts in c.c. of 1.15 molecular di-hydrogen pot. phosphate:—38, 43.5, 49.5, 55.5, 61.0, 66.5, 72, 76.5, 80.5, 84, 86.5, 89, 91.5, 93, 94.5 made up with 1.15 molecular di-hydrogen pot. phosphate to 100 c.c. to give, respectively, hydrogen ion concentrations of pH—6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, and 8.0.

**S 7.762.**—(1) Add to 25 c.c. N-5 di-hydrogen pot. phosphate (**S 7.74**) containing phenol red<sup>1</sup> the following amounts of N-10 sod. hydroxide:—17.8, 23.65, 29.63, 35, 39.5, 42.8, 45.2, 46.6 c.c. (2) Make up to 100 c.c. with water and so obtain mixtures of hydrogen ion concentration, pH 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8 and 8.

**Notes.**—<sup>1</sup>To prepare:—(i) weigh out 13.616 grm. di-hydrogen pot. phosphate ( $\text{KH}_2\text{PO}_4$ ) and dissolve in about 300 c.c. boiled D.W. (ii) Add 99.6 c.c. of 0.02 per cent phenol red. **S 7.775**.—<sup>2</sup>Make up the mixture to 500 c.c.

### S 7.77 INDICATOR SOLUTIONS.

**S 7.771.**—(1) Make a 1 per cent phenol phthalein in alc. of '937 specific gravity.

**S 7.772.**—(1) Make a 0.5 per cent phenol phthalein in 50 per cent alc.

**S 7.773.**—(1) Make a 0.1 per cent methyl orange<sup>1</sup> in D.W. (2)<sup>1</sup> Add dilute sulphuric acid carefully from a burette until the liquid turns red and just ceases to be transparent. (3) Filter.

**Notes.**—<sup>1</sup>Not affected by carbonic acid gas. Not usable with organic acids.

**S 7.774.**—(1) Make a 10 per cent pot. monochromate<sup>1</sup> sol. in D.W. (2) Add dilute silver nitrate sol. carefully from a burette until a slight red precipitate forms. (3) Allow the precipitate to settle. (4) Use the S.N.F. as indicator sol.

**Notes.**—<sup>1</sup>Used as indicator with silver nitrate titration for chlorides.

**S 7.775.**—(1) Dissolve 0.1 grm. phenol sulphone phthalein<sup>1</sup> in 100 c.c. D.W. to which 10 c.c. N-10 sod. hydroxide have been added. (2) Hasten the solution with gentle heating. (3) Add about 300 c.c. D.W. (4) Neutralize the alkaline sol. of the indicator exactly with 10 c.c. N-10 hydrochloric acid. (5) Make up the vol. to 500 c.c.<sup>2</sup>

**Notes.**—<sup>1</sup>Phenol red. <sup>2</sup>Useful range for titration pH 6.8 to 8.4; colour yellow to red.

**S 7.776.**—(1) Prepare 0.04 per cent brom thymol blue.<sup>1</sup> (2) Use to give a distinct grass green<sup>2</sup> colouration in titration.

**Notes.**—<sup>1</sup>Dibromothymol sulphone p' thalein. <sup>2</sup>This is approximately true neutrality—pH 6.9 to 7.4.

- S 7777.**—(1) Prepare a 0.5 per cent alc. sol. of thymol phthalein.  
(2) Use to give a simple darkening of colour<sup>1</sup> as compared with a control.

**Notes.**—<sup>1</sup>This simple darkening of colour takes place on the alkaline side of neutrality, pH 8.75 to 8.9, at 15°C.

- S 7778.**—(1) Mix equal parts 0.5 per cent china blue in aqueous sol. and 1 per cent rosolic acid in 95 per cent alc. to give an indicator sol.<sup>1</sup>

**Notes.**—<sup>1</sup>The colour turning point of this indicator is very close to pH=7; colourless or nearly so at pH=7, turning blue as the reaction becomes acid and red as it becomes alkaline. This indicator may be added directly to a nutrient medium, 1 c.c. per 100 c.c. and the reaction of the medium adjusted by titration of the medium or sample of medium directly. The indicator has no effect on the nutrient qualities of the medium.

- S 7779.**—(1) Use 1.6 per cent brom cresol purple<sup>1</sup> in 95 per cent alc. (2) Add 1 c.c. per litre of medium.

**Notes.**—<sup>1</sup>Purple in neutral or alkaline media, yellow in acid media: pH 5.2 to pH 6.8. Useful particularly to detect increase of acidity in sugar media.

### **S 779 INDICATOR SOLUTIONS.**

- S 7781.**—(1) Use 1.6 per cent cresol red in 95 per cent alc.  
(2) Add 1 c.c. per litre of medium.

**Notes.**—<sup>1</sup>Yellow in neutral or acid media, red in alkaline media. A very satisfactory mixture is given by mixing 0.5 c.c. each 1.6 per cent alc. sol. brom cresol purple and cresol red and adding this amount per litre of medium. This mixture changes very slowly from purple to yellow through a long range from about pH=8.0 to about pH=5.0. By comparing with a blank tube of the neutral medium it is very easy to detect an increase in either acidity or alkalinity.

- S 7782.**—(1) Mix equal parts 0.5 per cent china blue in aqueous sol. and 0.2 per cent phenol red to give an indicator sol.  
(2) Use in the same way as the combination china blue-rosolic acid (S 7778).

- S 7783<sup>1</sup>.**—(1) Grind up litmus in a mortar. (2) Add 5 vol. 90 per cent alc. (3) Boil on a water bath. (4) Decant the S.N.F. (5) Add 6 parts D.W. to the residue. (6) Boil. (7) Allow to cool. (8) Divide into two portions. (9) Render one portion slightly red with dilute sulphuric acid. (10) Add to this reddened portion the other untreated portion little by little until the mixture becomes blue again. (11) Filter through paper when cool. (12) Distribute into T.T. (13) Sterilize at 110C. (14) Keep<sup>2</sup> for use.

**Notes.**—<sup>1</sup>Sensitive litmus:  
<sup>2</sup>

**S 7784.**—(1) Grind up 50 gm. granulated litmus in a mortar. (2) Place in a well-stoppered bottle. (3) Add sufficient 90 per cent alc. to cover. (4) Leave 2 d. and agitate occasionally. (5) Pour off the alc. and add fresh until the decanted fluid is almost colourless. (6) Transfer the residue to a litre flask. (7) Add 1 litre D.W. (8) Steam 1 hour. (9) Allow to cool. (10) Decant the litmus sol. from the chalk which settles to the bottom of the flask. (11) Filter. (12) Sterilize 3 d. at 100°.

**S 7785.**—(1) Boil powdered litmus in 90 per cent alc. till no more red colour is extracted. (2) Digest the residue with cold D.W. (3) Pour off or filter off the fluid. (4) Boil the residue remaining with 5 times its weight of D.W. (5) Filter. (6) Preserve the filtrate.

**S 7786.**—(1) Prepare sat. aqueous sol. of litmus. (2) Add in quantity to the medium to give a distinct blue colour.

**S 7787.**—(1) Place in a well-stoppered glass bottle: solid commercial litmus 1; 95 per cent alc. 6. (2) Shake well once daily for 7 d. (3) Reject the alc. (4) Add fresh 95 per cent alc. (5) Shake well once daily. (6) Repeat the addition of fresh alc. until the alc. becomes only lightly tinged with violet on shaking up with the litmus. (7) Reject the alc. (8) Dry the alc. insoluble residue. (9) Make a sat. sol. of the dried residue in D.W. (10) Filter. (11) Dilute a portion of this sat. sol. with D.W. until its tint is a pure blue. (12) Add to this pure blue sol. very dilute sulphuric acid till the blue colour is turned to wine red. (13) Add to this wine red sol. the sat. sol. obtained from the alc. insoluble residue, until the blue colour returns. (14) Use as a sensitive litmus sol.

**Notes.**—Two drops dilute sulphuric acid to 200 c.c. water.

**S 7788.**—(1) Grind up granulated litmus. (2) Add 85 per cent alc. (3) Boil. (4) Add 7 parts water to the alc. insoluble residue. (5) Heat. (6) Filter through paper. (7) Add to one half of the filtrate sulphuric acid until the colour is nearly red. (8) Add the untreated half to it. (9) Distribute into T.T. (10) Sterilize at 115°C.

**S 7789.**—(1) Place solid commercial litmus in a flask. (2) Pour 96 per cent alc. over it. (3) Close the flask with a rubber cork. (4) Keep in an incubator at 30°C with shaking at intervals for 2 d. (5) Pour off the alc. and add fresh. (6) Continue the procedure until no more red violet colour is extracted by the alc. (7) Filter off the alc. (8) Dry the residue. (9) Dissolve in D.W. to saturation. (10) Add

sufficient 1-1,000 sulphuric acid to produce the slightest change in colour.

**Notes.**—<sup>1</sup>This sol. on being diluted with D.W. should give a pure blue colour.

### **S 7·79 INDICATOR SOLUTIONS.**

**S 7·791.**—(1) Prepare :—*a* naphthol phthalein 0·04 ; 60 per cent alc. 100.

**S 7·792.**—(1) Prepare :—Methyl red 0·02 ; 95 per cent alc. 100.

**S 7·793.**—(1) Prepare :— Acid fuchsin 0·5 ; D.W. 100. (2) Add N-1 sod. hydroxide drop by drop, shaking thoroughly, till the red colour disappears. (3) Use 1 per cent of this indicator in sugar media.

**Notes.**—<sup>1</sup>Very sensitive to acid formation, stable and without effect on bacteria growth. The adjustment of the reaction of medium to the indicator should be such that the former is distinctly pink when hot, but colourless when cold.

### **S 7·8 MEDIA.**

**S 7·81.**—(1) Place 5 c.c. medium in a porcelain evaporating dish. (2) Add 45 c.c. D.W. (3) Boil 1 min. (4) Add 1 c.c. phenol phthalein indicator sol. (**S 7·772**). (5) Titrate immediately with N-20 sod. hydroxide to give a faint but distinct pink colour as the end point. (6) Adjust the reaction of the medium to this point. (7) Add acid or alkali to obtain the desired reaction, and express it, with reference to the colour turning point of phenolphthalein in percentages of the normal acid or alkali added. (8) Use as a standard reaction, 1 per cent acid to phenol phthalein except in the case of sugar media where the reaction should simply be brought to the turning point of phenolphthalein.

**Notes.**—<sup>1</sup>In Eyre's scale, instead of being expressed as the amount of normal acid or alkali added per 100 c.c., it is given as the amount per 1,000 c.c. The plus sign is used when normal acid is added, after the adjustment to the colour turning point of the indicator, and the minus sign when normal alkali is added. In actual practice where the medium is already acid the adjustment by 2 steps, first to the turning point of the indicator and then to the required acid point is shortened to one step, by which the acidity of the medium is simply *reduced* to the required acid point. Thus, if 2·5 c.c. normal alkali per 100 c.c. medium are required to bring the medium to the reaction represented by the colour changing point of the indicator and the desired reaction is +1, i.e., 1 per cent acid to phenolphthalein, it would be sufficient to add, in one step only, 1·5 c.c. (2·5—1=1·5) of normal alkali per 100 c.c. medium to attain this result.

**S 7·82.**—(1) Use as reagents—phenolphthalein indicator sol. (**S 7·772**) ; N-20 sod. hydroxide ; N-1 sod. hydroxide ; N-20 hydrochloric acid. (2) Fill one 50 c.c. burette, which has been thoroughly cleansed and dried, with N-20 sod. hydroxide and another with N-20

hydrochloric acid. (3) Place 45 c.c. D.W. in either a 100 c.c. porcelain evaporating basin or in a 100 c.c. conical flask. (4) Add 5 c.c. of the test medium.<sup>1</sup> (5) Add 1 c.c. phenolphthalein indicator sol. (6) Mix thoroughly with a glass stirring rod. (7) Boil 2 min. and carry out the standardization at boiling temperature. (8) Add N-20 sod. hydroxide drop by drop while constantly stirring. (9) Continue until a faint, permanent, distinct pink colour is obtained. (10) Heat the sample again to the boiling point for 15 sec. and if the colour disappears, add immediately more of the N-20 sod. hydroxide to give the same end reaction. (11) Take this as the final reaction. (12) Calculate the reaction of the medium. (13) Adjust the reaction of the bulk medium by addition of the necessary amount of N-1 sod. hydroxide. (14) Mix thoroughly. (15) Steam the medium in the steam sterilizer; if it is to be subsequently sterilized at 100°C; in the autoclave if it is to be sterilized at a higher temperature. (16) Restore to original vol. (17) Titrate samples again. (18) Adjust the reaction of the medium again. (19) Return to the steam sterilizer 10 min. or boil for 5 min. over a free flame. (20) Restore to original vol. (21) Filter. (22) Carry the medium through the various steps to sterilization in T.T.

**Notes.**—<sup>1</sup>In the case of a medium containing gelatin or agar, heat the D.W. before adding the sample. <sup>2</sup>The reaction of the medium is calculated with reference to the turning point of the phenolphthalein indicator sol. If acid with respect to this point the plus sign is placed before the figure showing the percentage amount of normal acid present; if alkaline, the minus sign is placed before the figure showing the percentage amount of normal alkali present. <sup>3</sup>If this precaution is not observed, the final sterilization of the medium may cause a heavy precipitate of phosphates.

**S 7·83.**—(1) Make up 1·5 molecular di-hydrogen pot. phosphate<sup>1</sup> sol. by dissolving 27·231 gm. in D.W. and making up to 1 000 c.c. (2) Add 41·2 c.c. N-5 sod. hydroxide to 50 c.c. 1·5 molecular di-hydrogen pot. phosphate sol. and dilute with D.W. to make 200 c.c. = No. 1 sol. pH = 7·5. (3) Add 42·8 c.c. N-5 sod. hydroxide to 50 c.c. 1·5 molecular di-hydrogen pot. phosphate sol. and dilute with D.W. to 200 c.c. = No. 2 sol. pH = 7·6. (4) Add 44·2 c.c. N-5 sod. hydroxide to 50 c.c. 1·5 molecular di-hydrogen pot. phosphate sol. and dilute with D.W. to 200 c.c. = No. 3 sol. pH = 7·7. (5) Use phenol sulphone phthalein indicator sol. (**S 7·775**). (6) Measure into tube No. 1 of the colorimeter apparatus 5 c.c. No. 1 sol. and 0·5 indicator sol. (7) Measure into No. 5 tube, 5 c.c. No. 3 sol. and 0·5 c.c. indicator sol. (8) Measure into No. 4 tube, 5 c.c. water. (9) Add 10 c.c. hot test agar medium to 40 c.c. cold D.W. and mix well—1·5 agar. (10) Measure into No. 3 tube 10 c.c. of the 1·5 agar sol. before it has set and 1 c.c. indicator sol. (11) Measure

into No. 2 and No. 6 tubes 5 c.c. 1-5 agar sol. before it has set. (12) Add to the 1-5 agar sol. in No. 3 tube N-20 sod. hydroxide from a microburette<sup>2</sup> mixing<sup>3</sup> well, until the colour tint as seen in the colorimeter box<sup>4</sup> approaches that of No. 1 tube. (13) Note the amount of N-20 sod. hydroxide used. (14) Add half the amount of N-20 sod. hydroxide used to tubes No. 1 and No. 5.<sup>5</sup> (15) Continue the addition of N-20 sod. hydroxide to tube No. 3 until the tint is intermediate between that of Nos. 1 and 5 tubes, D.W. to equalize vol. being added to Nos. 1 and 5 tubes as the titration proceeds. (16) Melt the agar at intervals in boiling water as the titration proceeds, but have the containing tube well cooled before the colour comparison is made.<sup>6</sup> (17) Read off the total amount of N-20 sod. hydroxide required for 10 c.c. of the 1-5 test agar medium. (18) Add 25 times this amount of N-1 sod. hydroxide to each litre of the melted agar and mix thoroughly. (19) Distribute in T.T. (20) Sterilize 15 min. at 120C on 3 successive days.

**Notes.**—<sup>1</sup>KH<sub>2</sub>PO<sub>4</sub>. Acid pot. phosphate. <sup>2</sup>A 2 c.c. pipette graduated in 1-50ths. <sup>3</sup>By closing with the thumb and inverting several times. <sup>4</sup>This is a special box to hold 6 tubes and called a comparator. By means of it the tints of test medium and standard tints are easily compared. The six tubes are disposed in two rows of 3, No. 2 tube being directly behind No. 1 tube, No. 4 tube behind No. 3 tube and No. 6 tube behind No. 5 tube. <sup>5</sup>To keep the indicator at the same concentration as in other tubes. <sup>6</sup>The tint is much affected by temperature.

**S 7-84.**—(1) Have in readiness:—A set of tubes containing solutions of known pH with definite concentration of phenol sulphone phthalein indicator sol. (**S 7-761**) a colour comparator rack<sup>1</sup> for 6 tubes in 2 rows of 3, a microburette<sup>2</sup>, a pipette to deliver 0.5 c.c., an ordinary 5 c.c. graduated pipette, clean 'cordite' tubes (**S 7-7611**), phenol sulphone phthalein indicator sol. of 0.01 per cent made by diluting 0.02 per cent sol. (**S 7-775**) with an equal vol. of D.W., and N-20 sod. hydroxide sol. made by taking 500 c.c. N-10 sod. hydroxide plus 91 c.c. 0.01 per cent phenol sulphone phthalein indicator sol. and making the mixture up to 1,000 c.c. with D.W. (2) Add so much alkali in the form of N-1 sod. hydroxide or N-1 sod. carbonate to the test medium before adjustment of the reaction that a drop of the medium so treated on a piece of coralline paper<sup>3</sup> gives a faint rose tint. (3) Measure out 5 c.c. of the medium into one of the 'cordite' tubes. (**S 7-7611**) and add 0.5 c.c. of the 0.01 per cent phenol sulphone phthalein indicator sol. (4) Place this tube in slot No. 3 in the comparator rack. (5) Place in No. 2 and No. 6 slots the standard tubes giving those pH colour reactions between which it is desired

to adjust the reaction of the medium. (6) Place in No. 4 slot a tube of D.W. (7) Place in No. 1 and No. 5 slots tubes containing medium without the addition of any indicator sol. (8) Add slowly N-20 sod. hydroxide from the microburette to the tube of medium in No. 3 slot mixing well after each addition<sup>1</sup> until the tint is intermediate between those of the two standard tubes in No. 2 and No. 6 slots. (9) Calculate from the amount of N-20 sod. hydroxide added the amount of N-1 sod. hydroxide necessary to bring the bulk of the medium to this hydrogen ion concentration.

**Notes.**—<sup>1</sup>This is a special box called a comparator with slots for 6 tubes, open on front and back faces to allow of transmission of light. By means of it the tints of test medium and standard tints are easily compared. The 6 tubes are disposed in two rows of 3, No. 2 tube being directly behind No. 1 tube, No. 4 tube behind No. 3 tube, and No. 6 tube behind No. 5 tube. <sup>2</sup>A 1 or 2 c.c. bacterological pipette having a fine glass tip attached to it by rubber tubing and having delivery controlled with a pinch-cock. <sup>3</sup>Coralline or rose-red paper is prepared by dipping thin filter paper or glazed paper into 0.5 per cent sol. of the indicator in 50 per cent alc. The strips are then dried in the air and kept in a stoppered bottle. In using this paper the earliest change of colour can be detected by allowing the wet paper to come in contact with a piece of dry white filter paper which clearly shows the faintest pink colour. <sup>4</sup>This is necessary because the medium itself may have a tinge of colour. By placing a tube of medium behind the standard tubes this tinge of colour is superadded to the tint of the standard tubes and so comparability is attained throughout the system. <sup>5</sup>For strict accuracy the same amount of N-20 sod. hydroxide should be added at the same rate to the tubes in slots No. 5 and No. 6 so as to compensate for any colour change of the natural pigment of the medium. <sup>6</sup>For example:—a broth is required of pH=7.4. In order to bring the tint of a tube containing 5 c.c. of the medium to a tint which will match the standard 7.4 tube, say, 0.32 c.c. N-20 sod. hydroxide are used. Then the amount of N-1 sod. hydroxide required to be added to 2,000 c.c. of medium will be  $2,000 \times 1.20 \times 0.32 \times 1.5 = 64$  c.c. It will be noted that no allowance is made for the dilution of the bouillon by the 64 c.c. added. If the amount is not large, this error may be neglected for practical purposes.

**S 7.85.**—(1) Add to a small portion of the medium a few drops of brom thymol blue indicator sol. (**S 7.776**). (2) Note the colour produced. (3) Measure out 10 c.c. of the medium. (4) Place in a porcelain evaporating dish and dilute well with water. (5) Add 1 c.c. brom thymol blue indicator sol. (6) Place the porcelain evaporating dish under a burette containing N-10 sod. hydroxide. (7) Take the reading on the burette. (8) Allow the sol. to fall drop by drop into the porcelain evaporating dish, stirring between drops. (9) Continue the addition until the medium in the porcelain evaporating dish is a distinct grass green.<sup>1</sup> (10) Take the reading in the burette. (11) Determine the difference between the first and second reading.

(12) Calculate the amount of N-10 sod. hydroxide necessary to neutralize the medium exactly. (13) Adjust the reaction of the medium to this neutral point as given by brom thymol blue with N-1 sod. hydroxide.<sup>2</sup>

**Notes.**—<sup>1</sup>If too alkaline, the medium turns this indicator blue, if too acid, it becomes yellow, while, if of the correct acidity, it becomes grass green=pH 6·6—7·4. <sup>2</sup>The standardizing alkali need not be an exact normal solution or fraction of normal. All that is necessary is that the sol. which is used to adjust the reaction of the bulk of the medium shall be an exact multiple of the sol. used for titration.

**S 7·86.**—(1) Set up 2 series of 6 T.T. of approximately the same internal diameter,<sup>1</sup> one series containing 10 c.c. in each T.T. of dilute acid<sup>2</sup> sol. and the other series 10 c.c. of dilute alkaline<sup>3</sup> sol. (2) Add to the acid series of T.T. in order—0·9, 0·8, 0·7, 0·6, 0·5 and 0·4 c.c. phenol red indicator sol. (S 7·775). (3) Add to the alkali series of T.T. in order: 0·1, 0·2, 0·3, 0·4, 0·5 and 0·6 c.c. phenol red indicator sol. (S 7·775). (4) View together the acid T.T. having the larger quantity of indicator sol. with the alkali T.T. having the smaller quantity of indicator sol. and *vice versa* throughout the series pair and pair, to obtain colours corresponding to pH values 6·9, 7·2, 7·5, 7·7, 7·9 and 8·1 respectively. (5) Add 1 c.c. test medium to 4 c.c. D.W. in a T.T. of the same internal diameter as the pH series. (6) Add to this dilution of test medium 1 c.c. phenol red indicator sol. (7) Compare at this stage, if desired the colour of the test medium with the standard pH series to obtain the initial reaction. (8) Add N-20 sod. hydroxide sol. to the test medium dilution to give the required H-ion concentration, as given by comparison of the tint of the test medium with the tint afforded by conjunction of corresponding<sup>4</sup> acid and alkali T.T. (9) Read off the amount of N-20 sod. hydroxide used. (10) Adjust the reaction of the bulk of the medium to this point.

**Notes.**—<sup>1</sup>Measure an equal vol. of water into a number of T.T. and select those in which the water stands at about the same level. <sup>2</sup>One drop of concentrated hydrochloric or sulphuric acid per 100 c.c. D.W. is sufficiently strong. <sup>3</sup>The N-20 sod. hydroxide may be used or any sol. sufficiently alkaline to bring out the maximum red colour of the indicator. <sup>4</sup>Each pair of corresponding T.T. thus contains 1 c.c. of indicator sol. between them. The corresponding pair contains the larger amount of indicator sol. in the acid T.T. or the alkaline T.T. according as the pair of T.T. is to represent the acid or the alkaline end of the range of the indicator. <sup>5</sup>Fifty times the amount of N-20 sod. hydroxide should be added to 1 litre of medium. It is not obligatory that the titrating sol. should be exactly N-20 strength. If in carrying out the titration sufficient N-20 sod. hydroxide sol. is added to appreciably dilute the indicator sol., then the other tubes should be filled to a similar vol. before the final comparison is made. Comparison for the colour of fluids already having their own proper colour can be accomplished by placing a third row of tubes behind the pairs of acid and alkali tubes, containing 5 c.c. of the fluid itself.

**S 7-87.**—(1) Measure 10 c.c. test medium into a porcelain evaporating dish. (2) Add 25 c.c. pure D.W.<sup>1</sup> (3) Have a control dish alongside prepared in exactly the same manner. (4) Add 5 drops thymol phthalein<sup>2</sup> indicator sol. (**S 7-777**) to the first dish. (5) Run in N-10 sod. hydroxide from a burette, stirring continuously and looking for a darkening<sup>3</sup> of the colour as compared with that of the control dish. (6) Note the quantity of N-10 sod. hydroxide required to produce this change. (7) Take the average of several determinations. (8) Make a calculation of the amount of N-1 sod. hydroxide necessary to produce the same reaction in the rest of the test medium. (9) Add this amount to it and mix. (10) Bring this alkaline medium to the boil to deposit phosphates. (11) Filter free of phosphates. (12) Adjust the reaction by the necessary addition of the requisite<sup>4</sup> amount of N-1 hydrochloric acid to give a hydrogen ion concentration<sup>5</sup> of pH 7.6. (13) Control the reaction with cresol red and phenolphthalein (**S 7-772**) indicator solutions.<sup>6</sup> (14) Sterilize 20 min. at 115C.

**Notes.**—The addition of water does not appreciably alter the hydrogen ion concentration. After titration thymol phthalein is a most sensitive indicator. This indicator changes colour at a point on the ascending side of neutrality which ensures that sufficient phosphates will be thrown down to prevent their precipitation in the prepared medium. The objection has been raised that phenolphthalein and thymol phthalein indicators change colour at a point on the alkaline side of neutrality. But in bacteriological work this is really no objection, as it is necessary during the process of preparation to bring the meat extract to an alkaline reaction in order to get rid of phosphates. If one does not do so the broth will not be clear and the phosphates will be constantly precipitating out. <sup>3</sup>Just before this point a precipitate of phosphates occurs. The point may be more aptly described as the disappearance of the yellow tint and the development of bluish. Look for a darkening of the colour as compared with that of the control dish. <sup>4</sup>This amount has to be worked out for each particular type of medium by actual trial. <sup>5</sup>It has been found experimentally that the concentration of hydrogen ions in the purest water is, in terms of the 'normal' solution as standard,  $1 \times 10^{-7}$ . Hence the hydrogen exponent of water is 7. This represents true neutrality because in pure water the hydrogen and hydroxyl ions are equally balanced. The reaction of blood serum is in the region of pH=7.6. <sup>6</sup>To check the reaction in bulk it is not necessary to titrate out the broth again but only to show that it lies between 2 points. This can be done most conveniently by a suitable choice of indicators. Dilute 2 samples of 3 c.c. of the medium in T.T. with an equal quantity of D. W. Add to the first tube 2 drops of 0.5 per cent alc. phenolphthalein indicator sol. and to the second 2 drops of 0.02 per cent watery cresol red indicator sol. Fifteen per cent alc. may be added to the latter sol. as preservative. If the reaction is correct, no colour change should occur in the first tube while a rose to a pinkish colour should be gained in the second. Thus the reaction of the tested sample will not be less than pH 7.6, the point at which phenolphthalein first shows a trace of pink, but will be over pH 7.6, cresol red developing a rose pink (weak permanganate) at pH 7.6. If the reac-

colour does not appear but only a brown shade, the medium is on the acid side, while if a pink is obtained with phenolphthalein, the medium is too alkaline. The error in either direction can be adjusted by the addition of a few drops of N-1 sod. hydroxide or hydrochloric acid to the medium in bulk and the reaction again controlled.

**S 7·88.**—(1) Add a drop of the medium to a drop of a naphthol phthalein indicator sol. on a white tile till the first transient flush of bluish green occurs. (2) Read off the amount of N-1 sod. hydroxide used in bringing about this result. (3) Continue from this point with the addition of N-1 sod. hydroxide, using as indicator sol. 1 per cent phenolphthalein in abs. alc. until the first faint pink colour appears. (4) Read off the amount of N-1 sod. hydroxide used. (5) Take the mean of the two readings as giving the amount of normal alkali to use in the adjustment of the reaction.

**Notes.**—<sup>1</sup>0·04 per cent in 60 per cent alc.

**S 7·89.**—(1) Adjust the reaction to give a yellowish green to grass green tint with brom thymol blue, or maximum purple colour with brom cresol purple, or a fawn orange tint with phenol red, or a faint blue colour developing in the course of 10 to 25 sec. on red litmus paper in order to have a pH value of about 6·8 to 7·2.

#### **S 7·9 DISINFECTANTS.**

##### **S 7·91—B. COLI-PHENOL TEST.<sup>1</sup>**

**Note.**—<sup>1</sup>Various organisms have been used, *e.g.*, anthrax spores and *B. typhosus*. Other tests than those here described are likewise used, *e.g.*, thread test, garnet test, phagocytic test, iodine test, etc.

**S 7·911 STANDARD SUSPENSION** —(1) Use a standard peptone<sup>1</sup> bouillon<sup>2</sup> of reaction 1·5 per cent acid to phenolphthalein.<sup>3</sup> (2) Sow with *B. coli*. (3) Incubate 24 hours. (4) Shake well to break up clumps. (5) Filter with sterile precautions through a double layer of filter paper. (6) Use the filtrate as the standard suspension for tests.

**Notes.**—<sup>1</sup>*E.g.*, Witte's peptone or a brand giving equivalent results. <sup>2</sup>Composition and preparation (i) Prepare:—Lemco 20; standard peptone 20; sod. chloride 10; D.W 1000. (ii) Boil 30 min. (iii) Neutralize to phenolphthalein with N-1 sod hydroxide and make 1·5 per cent acid with N-1 hydrochloric acid. (iv) Filter and bring up to vol. 1000 c.c. (v) Sterilize. <sup>3</sup>The reaction should be given in terms of hydrogen ion concentration. This concentration may be set down at pH=7·6 provisionally.

**S 7·912 STANDARD PHENOL.**—(1) Use dry crystals of absolute phenol. (2) Make up by weight the dilutions of phenol which are used in the test fresh as required.

**S 7-913 STANDARD CULTURE MEDIUM.**—(1) Use 10 c.c. in each T.T. of standard medium<sup>1</sup> for testing the viability of the standard organism after its subjection to the action of disinfectant.

**Notes.**—<sup>1</sup>Sol. taurocholate 5; glucose 5; peptone 20; D.W. 1,000, tinted with pure litmus sol.

**S 7-914 TRIAL OF INHIBITION EFFECT.**<sup>1</sup>—(1) Set up in a rack 6 T.T. each containing 10 c.c. standard culture medium. (2) Sow each T.T. with *B. coli*. (3) Add immediately to each T.T. in turn 0.1 c.c. from a series of dilutions of the disinfectant. (4) Incubate. (5) Examine the 6 T.T. at intervals of 24 hours up to 7 d. (6) Note the tubes in which growth has taken place. (7) Determine the highest dilution of disinfectant which under the conditions inhibit<sup>2</sup> growth.

**Notes.**—<sup>1</sup>This preliminary to the test is often omitted. It is designed to determine the upper limiting quantity of test and standard disinfectants which, when added to a given vol. of nutrient fluid medium, will no longer suffice to inhibit the growth of the standard organism. <sup>2</sup>It may be taken for granted that, as the test is arranged, the effect of the highest dilution of disinfectant giving no growth is an inhibitory effect and not bactericidal.

**S 7-915 TRIAL OF STRENGTH.**<sup>1</sup>—(1) Set up a series of T.T. numbered 1, 2, 3 and so on. (2) Add 5 c.c. of each of a series of dilutions<sup>2</sup> in boiled D.W. of the test disinfectant. (3) Set up in racks T.T. containing 10 c.c. standard culture medium, and in sufficient number<sup>4</sup> for the test of viability of the standard organism after subjection to the action of the disinfectant. (4) Have in readiness a watch<sup>5</sup> or better a stop watch to indicate time intervals between the tests of 2½, 5, 7½, 10, 12½, 15, 20, 25 and 30 min. (5) Add, with sterile precautions, 0.1 c.c. *B. coli* standard suspension in turn to each of the T.T. containing disinfectant dilution, shaking each tube and allowing 15 sec.<sup>6</sup> between each separate operation of addition. (6) Remove with sterile precautions and in turn, a quantity of disinfectant suspension from each T.T. after the expiry of 2½ min. from the commencement of the experiment. (7) Add at once 0.1 c.c. of the quantity removed to each of the corresponding T.T. containing standard culture medium. (8) Repeat any surplus quantity removed. (9) Proceed in exactly the same fashion with the disinfectant-suspension mixtures at the intervals 5, 7½, 10, 12½, 15, 20, 25 and 30 min. from the commencement of the experiment. (10) Place all the inoculated standard culture medium T.T. in the incubator. (11) Examine for growth at intervals of 24 hours for 7 d. (12) Determine

from the result what should be the most satisfactory series of dilutions<sup>7</sup> to use in the actual test.

**Notes.**—<sup>1</sup>To determine the range of dilution of disinfectant which will give clear cut results of growth and no growth in the test. The range of dilutions of disinfectant required is one which includes a dilution strong enough to kill *B. coli* in  $2\frac{1}{2}$  min. and a dilution not strong enough to kill in 30 min. This trial may be replaced by the test itself. <sup>2</sup>The greatest concentration will be contained in No. 1 T.T., the next in No. 2, and so on. A convenient series for trial is 1-100, 200, 300, 400, 500. <sup>3</sup>The series for the *standard phenol* is determined in the same way and will not require repetition once it is determined. <sup>4</sup>These T.T. should be inscribed with the same number as the T.T. containing disinfectant to which they correspond. The time of contact of disinfectant and suspension should also be inscribed upon each. <sup>5</sup>An ordinary watch will serve the purpose if the experiment be started exactly at a  $2\frac{1}{2}$  or 5 min. graduation. <sup>6</sup>15 sec. should suffice for the completion of each separate operation. <sup>7</sup>Thus for example:—If the test disinfectant has killed throughout for all intervals from  $2\frac{1}{2}$  min. up to 30 min. at a dilution of 1-200, and shows its bactericidal effect only after an interval of 10 min. for the 1-300 dilution, the conclusion would be that a range of dilution of test disinfectant from 1-300 to 1-200 would afford the necessary clear cut results in the actual test. Such dilutions would be given, say, by the series 1-200, 225, 250, 275, 300.

**S 7·9·6 THE TEST.**<sup>1</sup>—(1) Set up 5 T.T. in series. (2) Add 5 c.c. of each of a series of 5 dilutions of the test disinfectant in boiled D.W. which the trial has shown to afford a satisfactory range. (3) Set up in racks T.T. containing 10 c.c. standard culture medium, and in sufficient number for the test of viability of the standard organism after subjection to the action of disinfectant. (4) Proceed further exactly as in the trial of strength for the determination of a suitable range of disinfectant (**S 7·9·15**). (5) Proceed exactly with the necessary standard phenol dilutions<sup>2</sup> as with those of the test disinfectant. (6) Calculate the phenol coefficient<sup>3</sup> from the result obtained.

**Notes.**—<sup>1</sup>The test is performed at a temperature between 62 and 67 F. <sup>2</sup>The dilutions of standard phenol may conveniently be .7, .75, .9, 1 and 1·1 per cent. <sup>3</sup>Divide the figures representing the percentage strength of the weakest lethal dilution of the phenol control by the figure representing the percentage strength of the weakest lethal dilution of the test disinfectant for both the  $2\frac{1}{2}$  and the 30 min. periods. The mean of the 2 results is the phenol co-efficient of the test disinfectant.

## **S 7·92 B. TYPHOSUS PHENOL TEST WITHOUT ADDITION OF ORGANIC MATTER.**<sup>1</sup>

**Notes.**—<sup>1</sup>As the standard suspension which is used in this test is a suspension in bouillon and contains therefore extract of meat and peptone, the description of the test as 'without addition of organic matter' is not strictly accurate. A standardized suspension (**S 7·32**) of organisms made from an agar slope in 0·85 S.S.S. is free from this objection, but is not usually employed in the test.

**S 7-921 STANDARD SUSPENSION.**—(1) Prepare a standard suspension for the test by subculturing *B. typhosus*<sup>1</sup> daily with a 4 mm. platinum loop for 7 successive days in standard culture medium.<sup>2</sup> (2) Have in readiness for the test a 24-hour culture thus prepared.<sup>3</sup> (3) Shake well. (4) Filter with sterile precautions through filter paper and use the filtrate as standard suspension. (5) Place the standard suspension when prepared in a water bath at 20C.

**Notes.**—<sup>1</sup>Strains of *B. typhosus* are considered to vary less in resistance than those of *B. coli*. <sup>2</sup>4 mm. internal diameter, wire of B.W.G. 27-28. An instrument is sold consisting of a set of metal rods for making loops of standard diameter. <sup>3</sup>Preparation:—(i) Place 500 grm. finely chopped beef steak in 1,000 c.c. T.W. (ii) Allow to stand 24 hr. in a cool place. (iii) Strain through cheese cloth by means of a tincture press until 1,000 c.c. fluid are expressed. (iv) Heat 1 hour in streaming steam. (v) Filter through paper. (vi) Make up to vol. to 1,000 c.c. (vii) Titrate. (viii) Adjust reaction to approximate neutrality to phenolphthalein with N-1 sod. hydroxide. (ix) Add 1 per cent standard peptone and 0.5 per cent sod. chloride. (x) Heat in streaming steam 30 min. (xi) Adjust reaction to pH 7.6. (xii) Filter through paper. (xiii) Fill into T.T. in quantities of 10 c.c. (xiv) Sterilize 15 min. at 15 lb. pressure.

**S 7-921. STANDARD PHENOL.**—(1) Prepare a stock standard phenol solution with boiled D.W. to contain exactly 5 per cent. pure<sup>1</sup> phenol by weight. (2) Make up the higher dilutions fresh each day for the test. (3) Shake up well the stock sol. before making dilution.

**Notes.**—<sup>1</sup>Merck's silver label or phenol of equivalent purity.

**S 7-922 THE TEST.**—(1) Prepare a 5 per cent dilution<sup>1</sup> of the test disinfectant with boiled D.W. (2) Make dilutions from this 5 per cent dilution up to 1-500 or higher.<sup>2</sup> (3) Place the dilutions of standard phenol and of test disinfectant in T.T. in quantities of 5 c.c.<sup>3</sup> and in serial order. (4) Set the T.T. in racks in a water bath at 20C. (5) Add 0.1 c.c. standard suspension every 15 sec.<sup>4</sup> to each dilution with a pipette graduated in 1-10th c.c. (6) Sow T.T., each containing 10 c.c. standard culture medium, with a 4 mm. platinum loop<sup>5</sup> from the series of disinfectant suspension mixture, at intervals of 2½, 5, 7½, 10, 12½ and 15 min. (7) Incubate 18 hr. (8) Read off the results and tabulate them for comparison. (9) Calculate the phenol co-efficient. (10) Calculate the cost of co-efficient per unit of disinfecting efficiency as compared with pure phenol=1.

**Notes.**—<sup>1</sup>This dilution should be made 24 hours before use in the test solution of disinfectant, sol. When the test disinfectant does not give a uniform 5 per cent solution, use a 1 per cent. <sup>2</sup>If greater dilutions of the test disinfectant than 5 per cent stock sol. than 1-500 are required, make them from a 1 per cent stock sol. <sup>3</sup>Use a

5 c.c. delivery pipette. (ii) Start with the strongest concentration of disinfectant and proceed to the weakest. (iii) Use the same pipette throughout, but wash it out with a portion of each weaker concentration before transferring the necessary quantum of that concentration to its T.T. <sup>4</sup>This is ample time. The number of disinfectant dilutions used must not be so great as to occupy in this operation the total  $2\frac{1}{2}$  min. required to complete one test. <sup>5</sup>At least 4 separate loops, and better 6, should be used. Not more than 15 sec. should be taken in the operation of transference of loop sample to standard medium. <sup>6</sup>Divide the figure representing the degree of dilution of the weakest lethal strength of the disinfectant by the figure representing the degree of dilution of the weakest lethal strength of the standard phenol for both the  $2\frac{1}{2}$  and the 15 min. periods. The mean of the 2 results is the phenol co-efficient of the test disinfectant. <sup>7</sup>(Cost of test disinfectant per gallon ÷ cost of carbolic acid per gallon) ÷ phenol co-efficient of test disinfectant. This follows from the fact that the phenol co-efficient of a disinfectant gives the strength of the disinfectant in terms of phenol and therefore the dilution in which it should be efficient.

### **S 7·93 B. TYPHOSUS—PHENOL TEST WITH ADDITION OF ORGANIC MATTER.**—(1) Place appropriate dilutions<sup>1</sup> of standard

phenol and of test disinfectant in T.T. in quantities of 4 c.c. and in serial order. (2) Set the T.T. in racks in a water bath at 20C. (3) Measure out 15 c.c. sterile stock organic matter into a large T.T. or flask and place in the water bath at 20C. (4) Add 1·5 c.c. standard suspension at 20C to the 15 c.c. stock organic matter and mix thoroughly. (5) Add 1·1 c.c. standard suspension organic matter mixture<sup>4</sup> every 15 sec. to each dilution of disinfectant and shake to mix. (6) Proceed as before to test the viability of the standard organism at intervals of  $2\frac{1}{2}$ , 5,  $7\frac{1}{2}$ , 10,  $12\frac{1}{2}$ , and 15 min.

**Notes** —<sup>1</sup>As 1 c.c. of organic matter is to be added to the 4 c.c. of dilution, the dilution will be increased 1·4th by this addition, *e.g.*, if the dilution is 1-200, it becomes 1-250. <sup>2</sup>Simply an amount to provide sufficient organic matter for the tests. <sup>3</sup>Standard peptone 10; gold label French gelatin 5; D. W. 100. Requires shaking up to mix, before use. <sup>4</sup>Represents 1 c.c. organic matter and 0·1 c.c. standard suspension. The alteration of dilution due to the addition of the 0·1 c.c. standard suspension is negligible.

### **S 8 STANDARDIZATION.**

#### **S 8·1 TRYPSIN.<sup>1</sup>**

**Note.**—<sup>1</sup>Required for control of preparation of 'trymedia' and for evaluation of antitryptic power of serum.

**S 8·11.**—(1) Dilute 60 c.c. formalin with twice its vol. of water. (2) Neutralize with N-10 alkali from a burette until the colour is just red to phenolphthalein indicator sol. (**S 7·772**). (3) Measure out 100 c.c. 4 per cent casein sol.<sup>1</sup> in 0·4 per cent sod. carbonate sol. into a small flask. (4) Raise the temperature of flask and contents to 40C. (5) Add 5 c.c.

test trypsin sol. to the flask. (6) Mix. (7) Remove immediately a sample of 25 c.c. from the digest mixture. (8) Keep the mixture at 40C. (9) Remove at intervals of 30, 60, 90 and 120 min. samples of 25 c.c. from the digest mixture. (10) Add to each sample, including the initial sample as it is removed, 30 c.c. neutralized formalin sol. (11) Titrate each sample as soon as ready with N-10 alkali against phenolphthalein indicator until the sol. has a distinctly red colour. (12) Note the amount of normal alkali used for each sample. (13) Calculate the degree of digestion.

**Notes.** —<sup>1</sup>A sol. of gelatin may be used. It should be a fairly thick sol. but must not set to a jelly on cooling. The gelatin sol., which is acid in reaction, must, like the formalin sol., be neutralized with N-10 alkali before use. <sup>2</sup>E.g., if a standard trypsin gives a net acidity at the end of 120 min. equal to 6 c.c. N-10 alkali, while a test trypsin only gives one of 3 c.c., the test trypsin is only half the strength of the standard trypsin. The acidity given by the initial sample before digestion has started must be subtracted from the total acidity at the end of 120 min. in each case in order to obtain the net acidity. The number of c.c. of alkali used to obtain net acidity is thus the index of degree of digestion.

**S 8'12.**—(1) Dilute 30 c.c. milk with 30 c.c. water in a small flask. (2) Raise the temperature of the flask and contents to 40C. (3) Add 3 c.c. test trypsin sol. (4) Keep the mixture at 40C. (5) Withdraw at minute intervals samples of 5 c.c. and heat to boiling point. (6) Note the exact time<sup>1</sup> at which curdling shows in the heated samples.

**Notes.** —<sup>1</sup>The activities of trypsin solutions are proportional to the time taken to produce curdling.

**S 5 13.**—(1) Prepare fresh:—Casein 1; N-10 sod. hydroxide 100. (2) Shake well. (3) Keep 30 min. at 45C. (4) Neutralize with N-10 hydrochloric acid. (5) Sterilize in the autoclave. (6) Set up 3 sets of 8 small T.T. in 3 rows containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 c.c. 1-5, 10, 15 test trypsin sol.,<sup>1</sup> one dilution for each row. (7) Add 2 c.c. casein sol. (8) Bring up the vol. to 3 c.c. with 0.85 S.S.S. (9) Keep 45 min. at 37C. (10) Add to each T.T. 4 drops alc. acetic acid.<sup>2</sup> (11) Determine in which T.T. and to what degree precipitation occurs.

**Notes.** —<sup>1</sup>Trypsin 0.5 gm.; N-1 sod. carbonate 0.5 c.c.; 0.85 S.S.S. 50 c.c. <sup>2</sup>Glacial acetic acid 1; abs. alc. 9; D.W. 9. <sup>3</sup>Glacial acetic acid 5; abs. alc. 45; D.W. 45.

**S 8'14.**—(1) Prepare:—Test trypsin 2; 1 per cent sod. bicarbonate 20. (2) Add 1 vol. test trypsin dilution thus prepared to 4 vol. milk. (3) Keep at 45C. (4) Take definite samples of the digest mixture every 5 min. and add to strong nitric acid. (5) Determine the point of time at which no coagulation shows with the nitric acid.

**S 8:15.** (1) Set up mixtures :—1 per cent suspension of test trypsin in varying amount with 1 c.c. 15 per cent gelatin at 37°C for 30 min. all the reagents being at 37°C before mixing. (2) Cool at the end of the 30 min. for 30 min. at 10°C. (3) Determine the smallest amount of trypsin causing complete liquefaction of gelatin : 1 unit of trypsin.

### **S 8:2 PIPETTES.**

**S 8:21.**—(1) Pipette mercury out of its containing bottle into a clean watch glass. (2) Transfer this mercury with a pipette to a succession of watch glasses to clean it thoroughly, leaving any dirty scum residue behind with each transference. (3) Take up 10 c.mm. of cleaned mercury into a standardized pipette. (4) Eject the measured 10 c.mm. into a clean watch glass. (5) Take up another 10 c.mm.<sup>6</sup> of cleaned mercury in similar fashion and add to the first 10 c.mm. 20 c.mm.<sup>2</sup> (6) Have in readiness as marking pen a hypodermic needle and Indian ink in a watch glass. (7) Tip the measured 20 c.mm. into the barrel of a capillary pipette.<sup>3</sup> (8) Hold a watch glass in the left hand and keep the tip of the pipette containing the 20 c.mm. of mercury in the watch glass.<sup>4</sup> (9) Manipulate the column of mercury down the stem to the extreme end of the pipette, keeping the pipette almost horizontal during the process and the watch glass in contact with the tip. (10) Place the watch glass carefully down on a sheet of white paper while still holding the pipette in the same relative position, taking care that the mercury does not run out of the pipette. (11) Dip the hypodermic needle held in the left hand into the Indian ink and mark the upper limit<sup>5</sup> of the mercury. (12) Tilt the mercury up the pipette until the lower limit of the column exactly coincides with the upper margin of the mark already made.<sup>6</sup> (13) Mark the new upper limit in the same way as before. (14) Carry the calibration up the capillary stem to give, say, 5 divisions of 20 c.mm.<sup>7</sup> each and a total column of 100 c.mm. (15) Tip out the mercury into the watch glass again or directly into the barrel of the next capillary pipette to be calibrated.

**Notes.**—<sup>1</sup>A certificated pipette, fitted with teat or rubber tubing. If the teat is too big, a small piece of rubber tubing may be drawn over the top of the pipette to enable the teat to get a hold. Use a hand lens to confirm by inspection of upper and lower limits the correctness of the amount taken up. <sup>2</sup>Any quantity desired may be taken as the unit for calibration. <sup>3</sup>Must be thoroughly dry. <sup>4</sup>So that, if the mercury runs out, it will be caught in the watch glass and can be put back again. <sup>5</sup>Begin contact with the glass well below the upper limit of the mercury and bring the charged needle carefully up to that limit. Thus the upper margin of the mark

made on the glass stem is the one to which to measure. If 20 c.mm. pipettes are all that are required, the necessary calibration will have now been accomplished. Fill the 20 c.mm. of mercury from the calibrated pipette into the barrel of another capillary pipette and continue in the same fashion to calibrate as many pipettes as are required. <sup>6</sup>Throughout these manipulations the tip of the pipette remains for safety sake still in the watch glass. <sup>7</sup>A pipette may be usefully calibrated simply in equal vol. without these vol. being necessarily of definite amount. <sup>8</sup>The first 100 c.mm. pipette thus made may be used as a standard pipette for measuring out that quantity of mercury for further calibration purposes.

**S 8'22.** (1) Use perfectly clean dry mercury. (2) Place an amount of mercury in the weighing pan of a chemical balance, judged to be slightly more than 1.37 grm. (3) Detach small particles of mercury from this amount by means of a piece of stiff paper folded, like a scoop, until the weight reaches 1.37 grm. exactly. (4) Use this weighed mercury for the calibration of 100 c.mm. capillary pipettes.

**Notes.**—<sup>1</sup>0.1 cc. mercury = 1.37 (the S.G. of mercury) × 0.1 grm. = 1.37 grm.

**S 8'23 DROP PIPETTE.** (1) Prepare the capillary pipette at the time of use. (2) Pass the capillary portion into a selected hole in a steel plate wire gauge. (3) Cut off the capillary flush with the upper surface of the gauge to obtain the dropping extremity. (4) Set up the pipette vertically for estimation of the drop number. (5) Allow the fluid under test to drop from the extremity of the pipette at uniform rate into a measuring receptacle accurately graduated at 1 or 2 c.c. (6) Determine the relative drop volume of various fluids for the gauge number selected.

**Notes.**—<sup>1</sup>Drops, from a clean pipette in the vertical position at a more or less constant rate, depend for their size solely upon the external diameter of the pipette at its extremity. <sup>2</sup>The barrel of the pipette will be of different capacity & length according as it is to be used as a hand dropping pipette or simply as an attachment nozzle to a burette. <sup>3</sup>This hole gives the measure for a given kind of the second or drop. <sup>4</sup>Once correlated to the size of drop, it serves for the manufacture of any number of standard drop pipettes. <sup>5</sup>This receptacle may be conveniently made out of wide glass tubing. It should have the graduation mark on a narrow neck, above which it is opened out to form a funnel. <sup>6</sup>The following is a list of relative drop volumes of a number of fluids. The smaller the number the larger the drop. Water 1; 0.85 S.S.S. 1; fresh human serum 1.1; inactivated rabbit serum 1.09; guinea-pig serum 1.06; cerebro-spinal fluid 1.02; peptone bouillon 1.2; standard agglutinable typhoid culture 1.2; *calc. Wassermann-antigen* 2.5.

## INDEX.

- ACETIC ACID.**—TEST TRYPSIN S8-13.  
**ACID.**—NORMAL S7-72.  
**ACIDITY.**—TEST TRYPSIN S8-11.  
**AGGLUTINABILITY.**—STANDARDIZATION S7-39.  
**ALBUMIN.**—URINE S7-1607.  
**S. ALBUS.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.  
**ALKALI.**—NORMAL S7-71.  
**AMINO ACID.**—TITRATION S8-11.  
**ANDRADE.**—INDICATOR S7-793.  
**ANIMAL.**—TEST STANDARDIZATION S7-38.  
**ANTIGEN.**—AGGLUTINABILITY STANDARDIZATION S7-39—AREA STANDARDIZATION S7-36—COLONY COUNT S7-34—DRIED S7-311—FILM STANDARDIZATION S7-35—FORMALINIZED S7-391—HÆMOCYTOMETER COUNT S7-33—LOOP STANDARDIZATION S7-37—M.L.D. STANDARDIZATION S7-38—POLLEN S7-5—STANDARDIZATION S7-3—TOXIN S7-4—TURBIDITY S7-1, S7-32—WEIGHT S7-31.  
**ANTIGEN SUSPENSION.**—STANDARD S7-911, S7-921—STANDARDIZATION S7-1203, S7-14, S7-15, S7-16.  
**ANTISEPTIC.**—STANDARDIZATION v. DISINFECTANT.  
**S. AUREUS.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.  
**BACTERIA.**—NUMBER S7-1203—WEIGHT S7-1203.  
**BACTERIAL.**—SUSPENSION v. ANTIGEN SUSPENSION.  
**BARIUM SULPHATE.**—STANDARD SUSPENSION S7-11, S7-12.  
**BARNETT AND CHAPMAN.**—TITRATION METHOD S7-86.  
**BLOOD.**—HÆMOGLOBIN ESTIMATION S7-2205.—TURBIDITY S7-1607.  
**BROM CRESOL PURPLE.**—INDICATOR S7-779, S7-89.  
**BROM THYMOL BLUE.**—INDICATOR S7-776, S7-85, S7-89.  
**BROWN H. C.**—OPACITY TUBES S7-12.  
**CARBOLIC ACID** v. PHENOL.  
**CASEIN.**—TEST TRYPSIN S8-13, S8-14.  
**M. CATARRHALIS.**—WEIGHT AND TURBIDITY S7-1203.  
**CAUSTIC SODA.**—NORMAL S7-713.  
**CHINA BLUE PHENOL RED.**—INDICATOR S7-782.  
**CHINA BLUE ROSOLIC ACID.**—INDICATOR S7-778.  
**V. CHOLERA.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.  
**COBRA VENOM.**—STANDARDIZATION S7-43.

**B. COLI.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.

**COLI-PHENOL.**—TEST DISINFECTANTS S7-91.

**COLONY.**—COUNT STANDARDIZATION S7-34.

**COMPARATOR.**—BOX S7-8304, S7-8401.

**CORALLINE.**—PAPER S7-8403.

**CRESOL RED.**—INDICATOR S7-781.

**DAHLIA.**—STAIN S7-3321.

**B. DIPHTHERIAE.**—TOXIN S7-41.

**DISINFECTANT**—AND ORGANIC MATTER S7-9201, S7-93 COLI PHENOL TEST S7-91—COST S7-9237 GARNET TEST S7-9101—INHIBITION EFFECT S7-914 IODINE TEST S7-9101—PHAGOCYTIC TEST S7-9101—PHENOL CO-EFFICIENT S7-9163, S7-9236—RIDEAL WALKER TEST S7-9—STANDARDIZATION S7-9—THREAD TEST S7-9101.

**DONALD.**—DROP PIPETTE S8-23.

**DREYER.**—STANDARDIZATION AGGLUTINABILITY S7-39—STANDARDIZATION TURBIDITY S7-13.

**DROP.**—PIPETTE S8-23.

**B. DYSENTERIAE.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.

**B. ENTERITIDIS (GÄRTNER).**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TURBIDITY S7-1203.

**ENUMERATION.**—BACTERIA S7-1203, S7-33.

**EYRE.**—SCALE S7-8101.

**FILM.**—STANDARDIZATION ANTIGEN S7-351.

**FORMALINIZED.**—ANTIGEN S7-391.

**FUCHSIN.**—INDICATOR S7-793.—STAIN S7-3321.

**GARNET TEST.**—DISINFECTANTS. S7-9101.

**GELATIN.**—TEST TRYPSIN S8-15.

**GENTIAN VIOLET.**—STAIN S7-3321.

**GONOCOCCUS.**—WEIGHT AND TURBIDITY S7-1203.

**HÆMOCYTOMETER.**—COUNT BACTERIA S7-33.

**HÆMOGLOBIN.**—ESTIMATION S7-2205, S7-23.

**HYDROCHLORIC ACID.**—NORMAL S7-723.

**HYDROGEN ION.**—COLOMETRIC SOLUTIONS—CONCENTRATION S7-8705, S7-76—STANDARDIZATION MEDIA S7-83 to S7-89.

**INDICATOR.**—ANDRADE S7-793—BROM CRESOL PURPLE S7-779—BROM THYMOL BLUE S7-776—CHINA BLUE PHENOL RED S7-782—CHINA BLUE ROSOLIC ACID S7-778—CORALLINE S7-8403—CRESOL RED S7-781—FUCHSIN S7-792—LITMUS S7-783 to S7-789—METHYL ORANGE S7-7222, S7-773—METHYL RED S7-791—NAPHTHOL PHTHALEIN S7-791—PAPER S7-8403—PHENOLPHTHALEIN S7-771, S7-772—PHENOL RED S7-775—POT. MONOCHROMATE S7-774—ROSOLIC ACID S7-778, S7-8403—SOLUTIONS S7-77 to S7-79—THYMOL PHTHALEIN S7-777.

**B. INFLUENZÆ.**—WEIGHT AND TURBIDITY S7-1203.

**IODINE TEST.**—DISINFECTANTS S7-9101.

**KUBEL AND TIEMANN.**—LITMUS SOLUTION S7-787.

**LO.**—DOSE S7-4112.

**L+.**—DOSE S7-4113.

**LITMUS.**—INDICATOR S7-783 to S7-789, S7-89.

**MAGNESIUM SULPHATE.**—TURBIDITY SUSPENSION S7-15.

**MEDIUM.**—LEMCO S7-9112 SOD. TAUROCHOLATE GLUCOSE S7-9131—  
STANDARD S7-9112, S7-913, S7-9213—STANDARDIZATION pH  
S7-83 to S7-89 STANDARDIZATION PHENOLPHTHALEIN  
S7-81, S7-82—STANDARDIZATION REACTION S7-8.

**M. MELITENSIS.**—WEIGHT AND TURBIDITY S7-1203.

**MENINGOCOCCUS.**—WEIGHT AND TURBIDITY S7-1203.

**MERCURY.**—CALIBRATION S8-21, S8-22.

**METHYL ORANGE.**—INDICATOR S7-7222, S7-773.

**METHYL RED.**—INDICATOR S7-791.

**MILK.**—TEST TRYPSIN S8-12.

**MINIMUM LETHAL DOSE.** STANDARDIZATION S7-38.

**OPACITY** v. **TURBIDITY.**

**NAPHTHOLPHTHALEIN.**—INDICATOR S7-791, S7-88.

**NITRIC ACID.**—TEST TRYPSIN S8-14.

**NORMAL.**—ACID S7-72—ALKALI S7-71.

**OXALIC ACID.**—NORMAL S7-721.

**ORGANIC MATTER**—AND DISINFECTANTS S7-9201.

**B. PARATYPHOSUS A.**—WEIGHT AND NUMBER S7-1203. —WEIGHT AND  
TURBIDITY S7-1203.

**B. PARATYPHOSUS B.**—WEIGHT AND NUMBER S7-1203.—WEIGHT AND  
TURBIDITY S7-1203.

**PHAGOCYTIC TEST.**—DISINFECTANTS S7-9101.

**PHENOL.**—COEFFICIENT S7-9163, S7-9236.—STANDARD S7-912, S7-922.

**PHENOLPHTHALEIN.**—INDICATOR S7-771, S7-772, S7-81, S7-82.

**PHENOL RED.**—INDICATORS S7-775, S7-83, S7-84, S7-86, S7-89—TUBES S7-761,  
S7-762.

**PHENOL SULPHONE PHTHALEIN.**—INDICATOR v. **PHENOL RED.**

**PHOSPHATE.**—PRECIPITATION IN MEDIA S7-8203, S7-8702—SOLUTIONS  
STANDARDIZATION S7-73 TO S7-75.

**PIPETTE.**—DROP S8-23.—MERCURY CALIBRATION S8-21, S8-22.—  
STANDARDIZATION S8-2.

**PNEUMOCOCCUS.**—WEIGHT AND TURBIDITY S7-1203.

**B. PNEUMONIÆ (FRIEDLÄNDER).**—WEIGHT AND NUMBER S7-1203—  
WEIGHT AND TURBIDITY S7-1203.

**POTASSIUM MONOCHROMATE.**—INDICATOR S7-774.

**POTASSIUM PHOSPHATE.**—MOLECULAR S7-73—NORMAL S7-74.

**B. PROTEUS.**—WEIGHT AND NUMBER S7-1203. —WEIGHT AND TURBIDITY  
S7-1203.

**B. PYOCYANEUS.**—WEIGHT AND NUMBER S7-1203—WEIGHT AND TUR-  
BIDITY S7-1203.

**ROSOLIC ACID.**—INDICATOR S7-778—PAPER S7-8403

**RIDEAL WALKER TEST.**—DISINFECTANT S7-9.

**SILT**—IN WATER S7-1607.

- SNAKE VENOM.**—STANDARDIZATION 87:43
- SODIUM CARBONATE.**—NORMAL 87:711, 87:712.
- SODIUM HYDROXIDE.**—NORMAL 87:713.
- SODIUM PHOSPHATE.**—NORMAL 87:84.
- SODIUM TAUROCHOLATE.**—GLUCOSE MEDIUM 87:9131.
- SOERENSEN.**—AMINO-ACID TITRATION 88:11—PHOSPHATE SOLUTIONS 87:73 to 87:75—TRYPTIC POWER 88:11.
- STANDARD.**—AGGLUTINATION 87:923—ANTITOXIN UNIT 87:1111—BACTERIAL SUSPENSION 87:911, 87:921—INDICATOR SOLUTIONS 87:77—LOOP 87:371, 87:9212—MEDIUM 87:9112, 87:913, 87:9213.—PHENOL 87:912, 87:922—SOLUTIONS 87:7.
- STANDARDIZATION.**—AGGLUTINABILITY 87:39.—ANIMAL TEST 87:38—AREA OF GROWTH 87:36—BACTERIAL ANTIGEN 87:3—BACTERIAL SUSPENSION 87:1203—COLONY COUNT 87:34—COLORIMETRIC pH 87:83 to 87:89—DISINFECTANTS 87:9—FILM 87:35—HEMOCYTOMETER 87:33—HYDROGEN ION MIXTURES 87:76—INDICATOR SOLUTIONS 87:7 to 87:9—MINIMUM LETHAL DOSE 87:38—NORMAL ACID 87:72—NORMAL ALKALI 87:71—OPACITY 87:1—PHOSPHATE SOLUTIONS 87:73 to 87:75—PIPETTES 88:21—PLATINUM LOOP 87:37—POLLEN 87:5—REACTION MEDIA 87:8—SNAKE VENOM 87:43—STANDARD SOLUTIONS 87:7—TINT 87:2—TOXIN 87:4—TRYPSIN 88:11—TURBIDITY 87:187:32—WASSERMANN TEST ANTIGEN 87:5—WEIGHT 87:31.
- SUGAR.**—URINE 87:1607.
- SULPHURIC ACID.**—NORMAL 87:722.
- B. TETANI.**—TOXIN 87:42.
- THIONIN BLUE.**—STAIN 87:332.
- THREAD TEST.**—DISINFECTANTS 87:9101.
- THYMOL PHTHALEIN.**—INDICATOR 87:777, 87:87.
- TINT.**—STANDARDIZATION 87:2.
- TITRATION.**—MEDIA 87:8.
- TINTOMETER.**—TUBES 87:22.
- TINTUROMETER.**—ESTIMATION TINT 87:23—ESTIMATION TURBIDITY 87:16—USE OF 87:16, 87:23.
- TOXOID.**—DIPHTHERIA 87:4111.
- TOXIN.**—B. DIPHTHERIE 87:41.—PURE 87:4111—SNAKE 87:43—STANDARDIZATION 87:4—B. TETANI 87:42.
- TOXONE.**—DIPHTHERIA 87:4111.
- TRYPSIN.**—ACIDITY TEST 88:11—CASEIN TEST 88:13, 88:14—GELATIN TEST 88:15—MILK TEST 88:12—STANDARDIZATION 88:1.
- TYPHOID-PHENOL.**—TEST DISINFECTANTS 87:92.
- B. TYPHOSUS.**—TURBIDITY AND NUMBER 87:14, 87:15—WEIGHT AND NUMBER 87:1203—WEIGHT AND TURBIDITY 87:1202.
- TUBERCULIN.**—STANDARDIZATION 87:381.

**TURBIDITY**—AND NUMBER S7·1203—AND WEIGHT S7·1203—ANTIGEN S7·1, S7·32—ESTIMATION S7·11, S7·12, S7·16—TINTUROMETER S7·16—TUBES S7·11, S7·12.

**UNIT**.—ANTITOXIN S7·4111.

**URINE**.—ESTIMATION ALBUMIN S7·1607—ESTIMATION SUGAR S7·1607.

**WASSERMANN TEST**.—ANTIGEN S7·6.

**WATER**.—ESTIMATION SILT S7·1607.

**WEIGHT**.—BACTERIA S7·1203, S7·31.

**WRIGHT**.—STANDARDIZATION ANTIGEN S7·342, S7·351.

ON THE SO-CALLED 'PENIS' OF THE BED-  
BUG (*CIMEX LECTULARIUS* L.) AND ON THE  
HOMOLOGIES GENERALLY OF THE  
MALE AND FEMALE GENITALIA  
OF THIS INSECT.

BY

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Received for publication, February 9, 1921.]

THE male and female external genital apparatus in the bed-bug has been described by several authors, notably Laudois( ) Patton and Cragg,(2) Rothschild,(3) Hay Murray( ) and Hase(4). The most complete description is that of Rothschild, who studied the parts with a view to utilising them in the differentiation of species. None of these authors, however, have given a satisfactory account of the homology of the different structures, and their descriptions are consequently somewhat lacking in enlightenment.

The terminal segments of the male in *Cimex*\* are modified in connection with the altogether extraordinary manner of copulation. The female has two sexual apertures, distinct and widely separate from one another, a copulatory orifice situated on the right side of the ventral aspect of the apparent fourth sternite just posterior to

\* NOTE.—We have dealt throughout with *C. lectularius*, but there is no reason to believe that the other species differ materially as regards the structures we have described.

the mid-point of the abdomen, and an orifice for oviposition in the normal ventro-posterior position. Moreover, the copulatory orifice of the female is of a special kind: there is a short longitudinal slit in the sternite and beneath this a solid structure, Berlese's organ, recently described in detail by Cragg, ( ) which has no aperture which could possibly admit the obvious male part. At the most the so-called penis of the male can be passed into the slit in the sternite. The so-called penis is claw-like, carried laterally and bent to the left, in correlation to the position of the copulatory orifice of the female and to the position taken up by the pair during the act of copulation. But there are obvious anatomical difficulties in the way of its use for the introduction of the spermatozoa into the organ of Berlese. A closer examination of this interesting structure reveals the fact that it is not the penis at all, but merely a grooved director along which the true intromittent organ glides during the act of copulation. By a comparison of the parts with those of other Rhynchota it has been possible to determine the homology of the organ. The sexually modified segments of the female with their appendages are also described and given their homologous denotation.

#### THE ABDOMEN IN CIMEX.

Before proceeding to discuss the sexually modified parts, it is necessary to define the taxonomy of the abdominal segments as a whole.

The abdomen in *Cimex* is composed of the following segments. There is an apparent first segment, consisting of both tergite and sternite, and carrying ventrally on each side well up towards its anterior edge a pair of spiracles. There follow five large unmodified, or but slightly modified, segments each carrying on the lateral portions of the sternite a pair of spiracles. Following the last of these and considerably smaller is an apparent seventh segment also bearing spiracles, but modified ventrally in the female in connection with the genital opening. The end of the abdomen is formed by the apparent eighth segment, which is without spiracles and is modified both in the male and in the female for sexual purposes. Particularly in the artificially distended bug can be seen a further minute ring-shaped segment, and beyond this at the anal orifice still another possible segment consisting of tiny hair-bearing plates resembling in appearance a minute tergite and sternite. (Plate XXX fig. 1).

It is certain that the first of the segments described above is not homologous with the true first abdominal segment in insects. Though this seems to us fairly obvious we have not seen it anywhere definitely stated in the description of *Cimex* that this is so. Later in this paper we give evidence for considering this segment to be in reality the second abdominal segment, and in the meantime, to avoid confusion, we shall in description give the segments their true homologous numbers, the apparent first being the true second and the minute ring-shaped segment the tenth with a possible eleventh segment represented by the two minute crescentic anal plates.

There is apparently no trace of the true first sternite, but what is almost certainly the remains of the tergite is to be seen as a crescentic portion of the large second tergite separated off by a distinct incrasation. In the nymph the existence of a first tergite is still more demonstrable, there being a distinct separate chitinisation representing this structure (Plate XXIX, fig. 7). In the nymph, as in the adult, no sternite is demonstrable. Comparative studies confirm the view that the crescentically marked off portion referred to is in reality the first tergite. In some bugs it exists as a distinct piece, but most frequently it is more or less fused with the second tergite the junction, however, being almost invariably clearly indicated by a deep sulcus or incrassation.

The second true segment in *Cimex* is very large and at the sides extends forwards in two blunt cornuate processes. Dorsally the chitinisation extends without a break across the body, there being no indication of separation into tergite and connexivum. Ventrally also the segment is continuously chitinised for its full width, but it is deeply impressed centrally, where the chitinisation is less intense, by the extension backwards of the metasternum and posterior coxae. Close up to the metasternum, almost at the anterior edge of the segment, are the spiracles.

Abdominal segments 3-7 are very similar to one another in shape and size. Each consists dorsally of a broad tergal plate, chitinised uninterruptedly from margin to margin, and a ventral area similarly chitinised across the whole width but less intensely in the middle. As in segment 2 there is no indication of separate connexival plates or of lateral pleural membrane. Each of these segments carries ventrally near its outer edge a pair of spiracles. The fifth segment has on the right side, in the female, a longitudinal slit extending the whole depth

of the segment, and leading to the structure known as the 'organ of Ribaga' which is the entrance to the 'organ of Berlese.' The so-called *penis* of the male is inserted into this slit during copulation. Dorsally between segments 3-4, 4-5 and 5-6 in the median line are indications of the invaginations usually termed stink glands.

The eighth segment consists dorsally of a continuously chitinated tergal plate differing only from those of the preceding segments in being smaller and more contracted owing to its approach to the termination of the abdomen. Ventrally in the female the segment is considerably modified, as described later in connection with the female genital opening; in the male there is a slight modification only which will be described when dealing with the male genital apparatus. On each side of the ventral surface of the eighth segment is a large and conspicuous spiracle. No trace of a spiracle is visible even on the closest examination on the succeeding segments. The presence of a spiracle on this segment and on no succeeding one is of importance. In almost all insects the last pair of spiracles are on the eighth segment; only in the Lepidoptera and Diptera are the last pair generally on the seventh segment. Exceptions in other orders than those mentioned are practically only seen when the eighth segment is obsolescent, and even in this case careful search may frequently show some mark or indication of the spiracle.\* The position of the spiracles is therefore additional evidence of the correctness of our interpretation of the segmental notation in *Cimex*.

The ninth segment is profoundly modified in both male and female. In both it forms the end of the abdominal box proper, the succeeding segments being loosely articulated and in life more or less telescoped within the body.

The tenth segment in the male is in the form of an almost complete chitinous ring without differentiation into tergite and sternite; in the female it is deficiently chitinated beneath. The small anal plates referred to previously are extremely characteristic of the Heteroptera; whether these are traces of an eleventh, or of the twelfth segment, or do not represent a true segment at all, we are unable to say. The tenth segment and the extension beyond it clearly form in *Cimex* the structure so characteristic of the Rhynchota generally and known as the *cauda*.

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\* A curious exception is the apterygote *Japyx*.

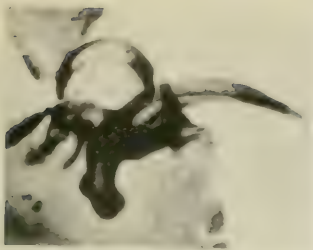
## PLATE XXIX.

- Fig. 1.** Partially dissected potash preparation of ♂ *C. lectularius* adult, showing the small ring-like tenth segment and the appendage. To the left of the base of the appendage is seen the chitinous framework of the phallosome. (Dorsal view.)
- " **2.** Potash preparation of ♂ *C. lectularius* adult, showing the ninth segment with the appendage, ring-like tenth segment, groove, etc. (Ventral view).
- " **3.** Potash preparation showing the phallosome dissected out. The heavily chitinised basal folds are conspicuous and around the organ is seen the delicate membranous capsule (membrane of genital cavity). Below is seen the delicate chitinous lining of the ejaculatory duct entering the organ and above the continuation of the phallosome as a delicate chitinous extension which in this specimen reached the full length of the groove in the appendage.
- " **4.** Ventral view of potash preparation of a ♂ Tingid Bug to show bilateral symmetry with two appendages and a large median phallosome as is usual in the Heteroptera.
- " **5.** Potash preparation of ♂ nymph showing ventral chitinous plates on abdominal segments 7, 8 and 9. The chitinous rudiment of the genital apparatus is seen behind the ninth segment which is undivided.
- " **6.** Potash preparation of ♀ nymph showing chitinous plates on abdominal segments 7, 8 and 9, the latter divided into two lateral portions, etc.
- " **7.** Dorsal view of abdomen of nymph showing presence of a first abdominal tergite. The last segment seen is the ninth as the small ring-like tenth segment is not shown. *Vide*, however, figs. 5 and 6.
- " **8.** Partially dissected potash preparation of ♀ adult showing the eighth and ninth segment with appendages. On the left side the lateral connexival piece of the eighth segment is seen with spiracle. Below are the two portions of the ninth sternite with their boss-like inner projections. Posterior to the hairy bosses lies the tenth segment. Anteriorly to the ninth sternal pieces are the narrow chitinous strips of the posterior gonapophyses.

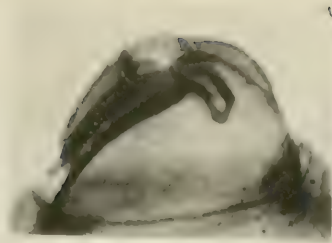
# PLATE XXIX.

- Fig. 1. Partially dissected potash preparation of *C. lectularius* adult, showing the small ring-like tenth segment and the appendage. To the left of the base of the appendage is seen the chitinous framework of the phallosome. (Dorsal view).
- " 2. Potash preparation of *C. lectularius* adult, showing the ninth segment with the appendage, ring-like tenth segment, groove, etc. (Ventral view).
- " 3. Potash preparation showing the phallosome dissected out. The heavily chitinised basal folds are conspicuous and around the organ is seen the delicate membranous capsule (membrane of genital cavity). Below is seen the delicate chitinous lining of the ejaculatory duct entering the organ and above the continuation of the phallosome as a delicate chitinous extension which in this specimen reached the full length of the groove in the appendage.
- " 4. Ventral view of potash preparation of a *C. Tingid* Bug to show bilateral symmetry with two appendages and a large median phallosome as is usual in the Heteroptera.
- " 5. Potash preparation of *C. nympha* showing ventral chitinous plates on abdominal segments 7, 8 and 9. The chitinous rudiment of the genital apparatus is seen behind the ninth segment which is undivided.
- " 6. Potash preparation of *C. nympha* showing chitinous plates on abdominal segments 7, 8 and 9, the latter divided into two lateral portions, etc.
- " 7. Dorsal view of abdomen of nymph showing presence of a first abdominal tergite. The last segment seen is the ninth as the small ring-like tenth segment is not shown. Vides, however, figs. 5 and 6.
- " 8. Partially dissected potash preparation of *C. adult* showing the eighth and ninth segment with appendages. On the left side the lateral connexival piece of the eighth segment is seen with spiracle. Below are the two portions of the ninth sternite with their boss-like inner projections. Posterior to the hairy pieces are the narrow chitinous strips of the posterior gonapophyses.

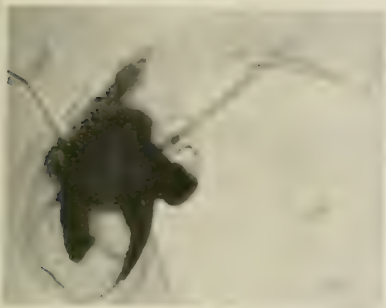
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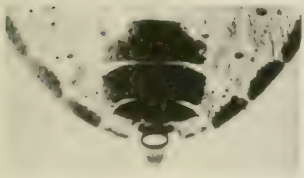
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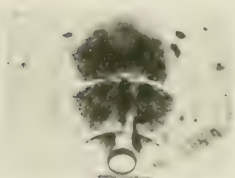
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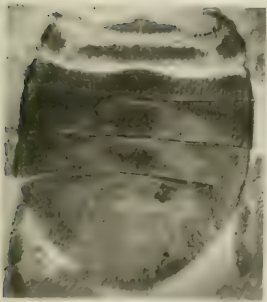
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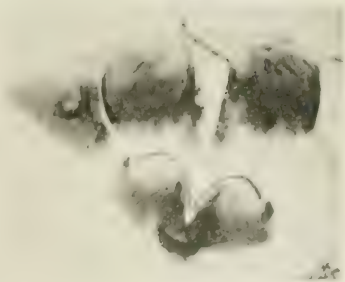
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In the nymph the presence of a distinct first tergite has already been referred to. The second tergite consists of a median transversely elongated chitinisation (tergite) and two lateral chitinised areas. The tergites 3-7 are much as in the adult; the third tergite is, however, unchitinised in its anterior portion. Ventrally the sternites are almost completely unchitinised, but there are median chitinous plates on the seventh, eighth and ninth segments and at the extreme lateral edge of each segment a small chitinous 'island.' In the fully grown nymph small chitinised spots also appear internal to the stigmata. In the young nymph, though the differentiation of the first five segments is well marked, the indications of the remaining segments dorsally are somewhat indistinct. In the last nymphal stage, however, the whole series of eleven abdominal segments composing the abdomen can be clearly made out. (Plate XXIX, figs. 5, 6 and 7).

#### THE GENITAL ARMATURE IN THE FEMALE.

The female opening is posterior to the eighth sternite. In connection with it the sternites of the eighth and of the ninth segments are modified.

The eighth sternite, instead of being continuously chitinised, as are the preceding segments, is subdivided into four separate plates, two lateral and two median. (Plate XXIX, fig. 8).

The lateral portions of the sternite carry, as already noted, well marked spiracles. Externally these portions are closely applied to the tergal plate without the intervention of a pleural membrane and form part of the sharp abdominal margin. There seems no doubt that these plates are homologous with the eighth sternal connexival piece to be seen in most Heteroptera, where they usually carry the eighth pair of spiracles.

The median portions of the sternite have been termed by Rothschild ) the *admedian plates*. These are entirely separated by a deep sulcus and soft membrane from the lateral pieces and from each other. Each plate is roughly quadrangular in shape, the posterior border, which is thin and somewhat rounded covering, in a resting condition, the lateral portions of the succeeding segment. From the internal border of the admedian plates spring a pair of processes shaped something like two quarters of an orange, the ventral surfaces, corresponding to the rind, being rather thickly chitinised and hairy. These processes lie almost directly over the opening of the common

oviduct and in dissections they often come away attached to this. The homology of the admedian plates and their processes is fairly simple. Throughout most of the Heteroptera a somewhat similar condition is seen, enabling their identity to be determined respectively as the halves of the eighth sternite and the processes commonly springing from these (*anterior gonapophyses, valvulae inferiores* of authors). As will be seen later, this view is confirmed by a study of the development. In *Cimex*, however, these processes are much reduced from their ancestral condition and lack characters which are to be found in those bugs where they are more developed and help to form an ovipositor.

The ninth segment completes the apex of the abdomen. In shape it is more or less of a triangular pyramid. Forming one side of the pyramid and completing the whole dorsal surface is the tergal chitinisation. The other two sides of the pyramid are ventral, and are formed respectively by the two separated portions of what for the time we may take to be the ninth sternite. These lateral sternal pieces are in most of their outer extent smooth and hairless, but their inner edges are raised to form conspicuous blunt hairy elevations. (Plate XXIX, fig. 8). Between these elevations lies a deep groove in which, at the extreme apex of the body, lies the minute tenth segment and the *cauda*. Anteriorly the groove is continuous with a hollow normally covered in and protected by the anterior gonapophyses, into which the oviduct opens.

Lying along the inner and anterior edges of the lateral sternal pieces of the ninth segment are the two peculiar lobes described by Rothschild. Each springs from a broad base and terminates in a narrow pointed extremity directed backwards and lying in the median genital groove. There seems little doubt that these are processes (*gonapophyses*) of the ninth segment or the *valvulae intermediae* of authors. The most striking peculiarity of these lobes is a long narrow line of chitinisation along their inner margin, which in a cleared preparation appears, as remarked by Rothschild, to be a long narrow free rod.

Chitinised strips are present on the gonapophyseal processes of many Heteroptera, both the anterior and posterior pair, and in less retrograde types form a groove and socket mechanism for linking these processes together. The strip in *Cimex* is therefore apparently a relic from an ovipositor bearing ancestor,

In *C. lectularius* these strips, it will be noted from the figure given, arise at the base close to, but not in direct continuation with, the lateral ninth sternal pieces. The explanation of this appears to be that normally in the Heteroptera the gonapophyseal chitinisation which they represent is a forward continuation of the outer angles of the median portion of the ninth sternite. The sternite in such cases is often divided into two lateral halves, widely separated, the central parts between these are depressed and, whilst wholly membranous in some forms, still exist as a chitinised plate or plates in others. In *C. lectularius* there is no central chitinisation and we may suppose that by disappearance of the median portion of the sternite the chitinisations of the gonapophyses have been left in a floating condition.

The third component part of the typical ovipositor *i.e.* the *gonapods*, *styles* or *calvae superiores* of authors are unrepresented in *Cimex*.

#### THE GENITAL ARMATURE IN THE MALE.

The eighth segment of the male in general characters resembles the preceding segments and shows no sub-division of the ventral surface into separate plates. The only modification in connection with the sexual function is a slight asymmetry and the presence of a depression on the left side at the apex, where the groove traversing the ninth segment ends and the tip of the so-called penis approaches it.

The ninth segment forms a somewhat flattened cap-like termination to the body. There is no separation into tergite and sternite, the dorsal and ventral surfaces being connected by continuous chitinisation so that the whole segment forms an almost completely closed box. The line of apparent junction of tergite and sternite is, however, depressed and forms a broad groove. This groove commences about midway on the right lateral border of the segment and, passing transversely around the apex of the body, continues along the whole length of the left side of the segment to terminate in the depression already referred to on the eighth segment. (Plate XXIX, fig. 2.)

Arising from an invaginated hollow in the floor of this groove a little to the right of the middle line of the body, is the so-called *penis*. This, at rest, is directed along the groove to the left passing under the tenth segment and *cauda*. Opening into the groove beneath the small tenth segment is, however, a second chitinous invagination in which lies

a structure so far not referred to by any author; this is the true intromittent organ or *Phallosome*.\*

The so-called penis is a claw-like structure formed of a continuous piece of chitin; it has been sufficiently described by Hay Murray and others. The most important feature is that it does not form a true canal, but is merely deeply grooved for a portion of its convex or posterior border. The phallosome is a complicated organ resembling in its structure the phallosome of other insects. In order to understand the nature of both these structures in *Cimex*, it is necessary to say a few words regarding the normal condition of the male parts in the order Heteroptera to which the bed-bug belongs.

In the Heteroptera, in accordance with the general plan of the male armature of insects, the ninth segment is specially modified. The modification most characteristic of the order is a tendency, often very pronounced, for the fusion of the tergite and sternite of this segment and the crumpling up of the posterior lip of the latter so that the whole segment forms a cup-shaped box. This chitinous box encloses a deep hollow, often opening externally only by a comparatively restricted opening, the *terminal chamber* of Sharp, or as we prefer to call it the *genital cavity*. Springing from the base of the genital cavity is a comparatively large non-segmental *phallosome*. On either side of the phallosome projects a structure composed of a single chitinous piece and usually more or less antler shaped. These antler-like structures arise from the inner aspect of the ninth sternite. They are termed by Sharp *lateral appendages*. In the Cryptocerata and some other forms they are seen as appendages arising from the edge of the sternite in the neighbourhood of the junction of the sternite with the tergite and lie externally. In some form or another, with a few exceptions, they are present throughout the Heteroptera and higher Homoptera. We have provisionally retained for them the name *lateral appendages*. The so-called penis of *Cimex* is one of the two lateral appendages normally

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\* No satisfactory term to indicate the complicated non-segmental intromittent organ as a whole appears to exist. Sharp(?) dealing with the male organs in the Pentatomidæ, speaks of the *aedoeagus*, but apparently does not include in this term the *theca*, an essential and important part of the non-segmental mass. Edwards(8) uses *aedoeagus* for the parts in the mosquito, but the same term is used in a restricted sense in the Lepidoptera. As it is often convenient to signify the whole non-segmental mass without implying homologies other than in the organ as a whole we have used the term *phallosome*, which is in keeping with the nomenclature now coming into general use and is devoid of ambiguity.

present in the order, the other appendage having disappeared. Owing to the distension of the parts in *Cimex* it is not possible by inspection to be quite certain which of the appendages has developed and which has disappeared, but a study of the development (*vide* next section) shows that it is the left appendage which has become the body hitherto called the penis.

The *phallosome* springs in the middle line from the membrane between the ninth and tenth sternites. It is usually a comparatively large organ and extremely complex in structure. Essentially it consists of (1) a basal fold very heavily chitinised and most often shaped remarkably like a stirrup; (2) the portion called by Sharp, the *theca*; (3) a median organ carrying the opening of the ejaculatory duct.

The massive stirrup-shaped piece is highly characteristic of the order and occurs in more or less recognisable form in practically all bugs. It may be regarded as a circumphallic chitinisation of the membrane of the base of the genital cavity, encircling the base of the phallosome and usually forming a bed for its reception. By it the whole organ is slung and it is to this body that the powerful muscles of the part are attached. For the time we may call it the *basal fold*.

The *theca* is also present throughout the order. An important part of the theca is the inversely reflexed fold of membrane lining its cavity. This is frequently eversible and may be furnished with most complicated saccular dilatations with chitinous thickenings and processes.

The third portion of the phallosome may probably be correctly homologised as the *mesosome*. Sharp refers to it along with certain lateral processes as the *aedwagus*. This is essentially a papilla carrying the opening of the ejaculatory duct, the outer layer being continuous below with the lining membrane of the theca. When the parts are fully extended the mesosome sometimes carries with it this lining membrane with all its accessory parts (if present) and there may thus be formed a large compound eversible sac.

In *Cimex* the phallosome has the appearance of this organ in bugs generally, though it is much reduced in relative size and complexity. It lies in a sac-like envelope, the membrane of which is continuous with the external chitin of the body, the cavity opening by a narrow neck, under the tenth segment. This sac appears to be the greatly reduced *genital cavity* or hollow of the ninth sternite, here reduced out of all resemblance

to its original condition. On the sides of this saccular envelope, closely embracing the phallosome, are two peculiar thick chitinous folds (Plate I, figs. 1 and 3), very conspicuous both in potash preparations and in sections. The appearance of these suggests the *basal fold* of other Heteroptera. Of these folds one lies to the right and somewhat dorsally and the other, which is somewhat larger, to the left and more ventrally. Partly enveloped by these folds is a thick basal portion of the phallosome springing from the base of the cavity and receiving the common ejaculatory duct. This we take to be the *theca*. Finally there is a soft terminal portion, capable of extension and covered in part with minute chitinous teeth; this we take to be the *mesosome*. In sections the ejaculatory duct after entering the base of the phallosome is seen to form a dilatation. This appears to be followed by a complicated folding within the thecal portion and then by a straighter portion lying in the papillary projection we have called the mesosome.

As ordinarily seen in dissections or in sections of the parts, the mesosome is a flaccid papilla, which at the neck of the phallosomic capsule is directed somewhat to the left and comes practically to lie upon (dorsal to) the basal portion of the *appendage* and just about reaches the commencement of the groove in this structure. In some cases it is found extending the whole length of the groove and can be pulled out from this. There can be little doubt that in the act of copulation, the *appendage* having been inserted into the cleft in the fifth sternite, the mesosome passes along the groove until it comes into relation with the so-called organ of 'Ribaga,' through which the spermatozoa enter the body of the female.

#### DEVELOPMENT OF THE GENITAL STRUCTURES IN CIMEX.

##### *External indications of sex in the nymph.*

From the earliest stage in the nymph it is possible, in a suitable preparation, to distinguish the male from the female.

In the nymph the ventral surface of the abdomen is mainly unchitinised. There are, however, on the seventh, eighth, and ninth segments rather conspicuous median chitinous plates. These plates, especially that on the ninth segment, differ in shape and size in the two sexes. In addition, especially in the last instar, there are certain indications of the future genital structures of the adult,

In the female the plate on the eighth segment shows posteriorly in the middle line a notch, on either side of which is a differentiation of the chitin suggesting a fore indication of the anterior gonapophyses. The ninth plate abuts closely upon the base of the tenth segment. In the last instar this plate consists of two lateral triangular portions separated by a median area of smooth chitin. The lateral portions have the appearance of separated halves of the sternite, the central portion being interpolated between these. The central portion is more or less deficiently chitinised and there is usually to be made out on either side, close to the middle line, a circular or oval pale spot. (Plate XXXI, fig. 9.) These spots as will be seen are indications of the gonapophyses of the ninth segment.

In the male the eighth plate is unmarked by any notch. The ninth plate is also entire, but at its posterior border and separating this from the tenth segment is a chitinous complex of peculiar character. This is indicated even in early instars, but is most distinctly developed in the last instar where it has the appearance shown in Plate XXXI, fig. 8 (*vide* also Plate XXIX, fig. 5).

Both in the male and in the female, these chitinous appearances are, as will be seen, the cuticular expression of early developmental changes, the nature and significance of which will be seen later.

#### *Development of the parts in the female.*

A complete description of the development of the sexual organs in the female is reserved for a future communication by one of us. For the present it is only necessary to refer to such portions of the development as will throw light on the nature of the parts we have so far described in the adult and in the cuticle of the nymph.

Indications of the future parts are clearly seen at the commencement of the last instar, as shown in Plate XXXI, fig. 6. Beneath the eighth sternal plate of the nymphal cuticle is the epidermal layer which will form the eighth sternite of the adult. From the posterior edge of this epidermal forecast of the future eighth segment there projects backwards on either side of the middle line, a flattish projection. As development proceeds these projections gradually assume the appearance and character of the processes we have termed the anterior gonapophyses.

Posteriorly to the eighth sternite the epidermal layer that will form the ninth sternite becomes differentiated into a central depressed portion and two lateral raised areas. These latter lie under

(and at the penultimate ecdysis are coterminous with) the divided chitinous sternal plate of the nymph. The lateral areas become eventually the inner raised and hairy portions of the plates in the adult which we have termed the lateral sternal pieces. The depressed central portion of the ninth sternite (if it is not more correctly considered as intersegmental membrane exposed by the drawing apart of the halves of the sternite) is sharply demarcated laterally by the abrupt and even overhanging edges of the lateral sternal areas and is anteriorly continued deeply under the overhanging eighth sternite with its processes. Arising from this depressed portion, on either side of the middle line, are early seen two flat projections, the rudiments of the gonapophyses of the ninth segment. This is the stage shown by the cuticle of the last instar nymph, as will be seen by comparing fig. 6 with fig. 9 in Plate XXXI. At this time the structures lying posterior to the ninth segment are exposed but later the hinder edge of the eighth sternite with its appendages comes more and more to project over and roof them in, forming at the same time a large intersegmental cavity.

Following the penultimate ecdysis there appears between the posterior pair of gonapophyseal processes a median longitudinal groove, the rudiment of the invagination which will form the common oviduct. This groove appears first as a narrow furrow. As development proceeds the furrow becomes a shallow flat invaginated cavity, which burrows its way forwards whilst its opening at the same time widens out. Eventually, whilst the invaginated undermining portion reaches the oviducts, the opening has become so extended that it practically occupies the whole of the dorsal aspect of the original intersegmental cavity formed by the continued growth of the eighth sternite and its appendages. The edges of the furrow have now been carried so far laterally that they reach the position which in the adult is marked by the bases of the chitinous strips on the gonapophyses. These processes, which we have seen are at first situated near the middle line, are carried by the widening of the oviductal margins far to the side; it is this which causes them to appear in the adult as appendages from the lateral portions of the ninth sternite. We have already seen that they originate as projections from the median depressed area.

As regards the common oviduct it is evident that there is considerable complexity in the composition of this apparently simple structure as seen in the adult. There are represented three separate cavities: (a) the original cavity of the intersegmental space; (b) the cavity formed by the

growth of the oviductal furrow; (c) an upper pouch arising from (b) and forming a Y-shaped extension reaching to the oviducts. (Plate XXXI, figs. 7, 0", 0' and 0, respectively.)

*Development of the parts in the male.*

Briefly the generative organs in the male are as follows. Passing backwards from the *testes* are the *vasa deferentia*. These are swollen towards their lower ends and act as *receptacula seminales*. Lying external to the lower ends of the vasa deferentia are the accessory glands with their ducts. At a level with the hinder end of the eighth segment the vasa deferentia along with the ducts of the accessory glands enter a muscular organ of conspicuous nature, the *sacculus*. Immediately posterior to the sacculus is the wide short *ductus ejaculatorius*. The arrangement of parts at the junction of the sacculus and ductus is peculiar, since from the former a kind of papilla or *callosum* projects into the lumen of the latter. On the surface of this papilla the four ducts of the vasa and accessory glands, respectively, open simultaneously. The ductus ejaculatorius, which has an obvious chitinous lining and is of large lumen, enters, after a short course, the base of the phallosome and comes in relation with the parts we have already described.

At an early stage of development there are present at the posterior border of the ninth sternite two bilaterally arranged blunt epidermal outgrowths (*primitive projections*) which spring from the dorso-anterior wall of a shallow invagination behind the sternite (*genital cavity*). The outgrowths fully occupy this cavity and project beyond its rim (Plate XXXI, fig. 1). On the anterior wall of the genital cavity between the bases of the primitive projections is a small pocket-shaped invagination, the first rudiment of the *ejaculatory invagination*. This is the stage represented by the cuticular parts of the last nymphal instar. The cuticular parts of the last nymphal instar are shown in Plate XXXI, fig. 8. It will be seen by a comparison of this figure with that giving the early epidermal condition (Plate XXXI, fig. 1) that the chitinous structure situated posteriorly to the ninth sternite in the nymph, at first rather puzzling, really consists of (a) the edge of the genital cavity; (b) the primitive epidermal processes; (c) the early indications of the ejaculatory invagination, all as they exist at the end of the penultimate instar. At the time of the penultimate ecdysis, however, there is considerable lateral expansion and some flattening of the parts as

will be seen from the figures which are drawn to the same scale. Already at this stage the chitin shews some differentiation of the inner portions of the primitive projections.

At a later stage there appears a fissure which starts posteriorly and dorsally and finally divides off on each side an inner portion of the primitive projection from an outer. Thus a fold is developed on either side of the rudimentary ejaculatory depression. These folds ultimately develop into the phallosome, the outer portions of the primitive projections becoming the appendages. The phallosomic folds at a later stage become a conspicuous median structure projecting from the ventral surface of the body anterior to the tenth segment. This structure is at first perfectly symmetrical, somewhat spout-shaped and with a deep groove along its whole length ventrally. This groove anteriorly is continued into a comparatively large globular sac, the now much enlarged ejaculatory invagination. The epidermal projections forming the rudiments of the future appendages lie symmetrically on either side of the rudiment of the phallosome and are at first similar in size and shape (Plate XXX, fig. 4, and Plate XXXI, fig. 3)

As development proceeds and the phallosomic rudiment increases in size it comes to lie somewhat asymmetrically. The left appendage also becomes larger than the right and exhibits at its outer angle a sharpish projection, the first indication of the blade portion of the penis (Plate XXXI, figs. 4 and 5). A fold is now present on either side of the phallosome lying between this and the rudiments of the appendages. This becomes the structure we have termed the *basal fold*. Later stages of development show the originally grooved phallosomic rudiment as a tubular organ. It appeared to us that this transformation from a grooved to a tubular structure takes place by the blending of the lips of the groove, and in some sections there appears to remain after closure of most of the groove into a canal, a still patent opening at the base where a depression is shown in Plate XXXI, fig. 5. This, however, equires confirmation. The phallosome has now very much the characters of this structure in the adult. It is of considerable relative size, however, and in this respect resembles the phallosome as usually seen in the Heteroptera.

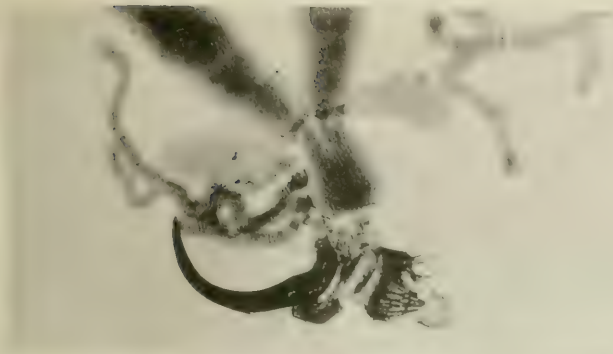
By the time the phallosome has reached this stage, the appendages have become markedly different in character. The right appendage is of its original size and shape; but the left appendage has greatly increased in size and has extended beyond the border of the genital cavity as a

# PLATE XXX

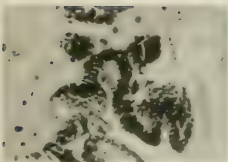
- Fig. 1. Dissection showing, above and in the centre, the vasa deferentia and their entrance into the sacculus. Laterally are the dilatations and glandular portions of the accessory glands. Below on the right is the tenth segment with the extended cauda. On the left is the appendage. Between the tenth segment and the appendage is the minute phallosome with its chitinous basal folds. Between the phallosome and the sacculus can be seen the transparent ductus ejaculatorius. The four ducts in the sacculus can be made out.
- .. 2. Horizontal section through caudal extremity of male nymph near penultimate ecdysis. This shows in the centre the primitive ejaculatory invagination and above this, on either side, the rudiments of the accessory glands, etc. Below on the right one of the two primitive projections is cut through the section being somewhat oblique has missed that of the opposite side.
- .. 3. As in fig. 2, but shortly after penultimate ecdysis. In the centre is seen the ejaculatory invagination and on either side of this is the fold which will eventually form the phallosome.
- .. 4. Transverse section of a later stage showing formation of the scoop-like rudiment of the phallosome and on either side in transverse section the appendages. The cavity in which they lie is the genital cavity.
- .. 5. Section at about the same stage but cut horizontally to show globular ejaculatory invagination and plaque. The phallosome is cut longitudinally through its lateral lips. A section or two further on the opening to the ejaculatory invagination is reached.
- .. 6. Section through the U-shaped loops forming the rudiments of the vas deferens and accessory glands. The ejaculatory invagination is just missed, but a few cent. of its wall is cut away and between the bends of the loops. On the right the continuation of the inner arm of the loop into the sacculus can be seen.

# PLATE XXX.

- Fig. 1. Dissection showing, above and in the centre, the *vas deferens* and then entrance into the *sacculus*. Laterally are the dilatations and glandular portions of the accessory glands. Below on the right is the tenth segment with the extended cauda. On the left is the appendage. Between the tenth segment and the appendage is the minute phallosome with its eversible basal folds. Between the phallosome and the *sacculus* can be seen the transparent ductus ejaculatorius. The four ducts in the *sacculus* can be made out.
2. Horizontal section through caudal extremity of male nymph near penultimate ecdysis. This shows in the centre the primitive ejaculatory invagination and above this, on either side, the rudiments of the accessory glands, etc. Below on the right one of the two primitive projections is cut through the section being somewhat oblique has missed that of the opposite side.
3. As in fig. 2, but shortly after penultimate ecdysis. In the centre is seen the ejaculatory invagination and on either side of this is the fold which will eventually form the phallosome.
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6. Section through the U-shaped loops forming the rudiments of the *vas deferens* and accessory glands. The ejaculatory invagination is just missed, but a few cells of its wall lie just below and between the heads of the loops. On the right the continuation of the inner arm of the loop into the delicate genital cord can be seen.



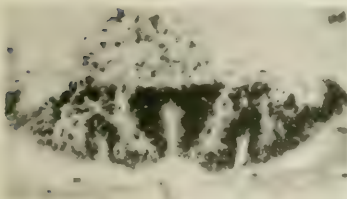
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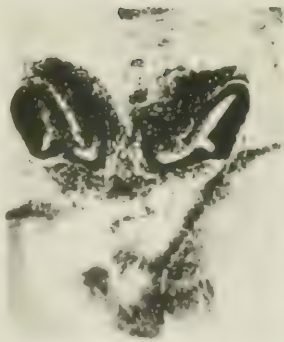
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long blade-like organ, on which can be clearly seen the commencement of the formation of the groove characteristic of this structure in the adult. The extra-genital portion of the left appendage lies at this stage upon the outer surface of the epidermal layer of the ninth segment of the future adult in a position external to the chitinous plate of the nymph. Its presence causes a sulcus to be formed in this situation, which eventually becomes the groove in which the so-called penis lies. This groove has not therefore, in its early stages at least, any very direct relation to the line of junction of tergite and sternite. Where the appendage leaves the genital cavity its presence causes the edge of this cavity to be somewhat flattened out, and at this point (the left angle of the genital cavity rim) very early chitinisation occurs so that in the newly emerged adult there is seen in this situation a dark V-shaped chitinisation even when the remaining parts are mostly still soft and uncoloured.

The further development of the ejaculatory invagination and of the rudiments of the vasa deferentia and accessory glands which become associated with it may be briefly mentioned, especially as this is of considerable interest and demonstrates very clearly the origin of most of the male genital structures we have mentioned at the commencement of this section. At the stage when the primitive projections are still undifferentiated there are seen, one on either side of the quite small ejaculatory invagination, the early common rudiments of the vasa deferentia and accessory glands. At this stage these are hollow, almost spherical dilatations at the terminations of a tube on either side passing backwards from the developing testes. These bodies are quite independent of the ejaculatory invagination of the epidermal layer and appear not to be epidermal in origin. At a later stage the original more or less globular vesicles become elongated and bent upon themselves forming on either side a U-shaped tube with the convexity of the loop directed posteriorly. These tubes lie in the eighth segment. The inner limb of each is continued as the delicate tubule we have already referred to (genital cord), and is evidently the rudiment of the vas deferens. The outer limb forms a thick walled sac ending blindly. This sac early begins to take on the form and characters of the accessory gland complex. At this stage the vas deferens and accessory gland of each side form parts of a single tube, their cavities being continuous through the bend of the loop.

The rudiments lie at a little distance from the ejaculatory invagination, which, by the time the vas deferens and accessory gland portions have begun to be differentiated, has become a large and conspicuous globular sac, on the floor of which has been developed a peculiar thickened cellular plaque (Plate XXX, fig. 5). The lower ends of the U-shaped loops as development proceeds grow towards and finally enter this plaque. We have not actually seen the loops reach the surface of the plaque, but this no doubt occurs with the eventual opening of their lumen and the formation of four separate ducts. Muscular elements appear around the loops as they grow towards the plaque and in this way is formed the muscular sacculus. The short wide ejaculatory duct is the original globular epidermal invagination; the velum is the cellular plaque on the floor of this cavity.

We have already stated that the rudiments of the vasa deferentia and accessory glands have no connection with the epidermal invagination forming the ejaculatory duct, and that they arise in connection with the testes. That they are not of epidermal origin is further shown by the fact that in the adult a chitinous lining is demonstrable after treatment with caustic potash only so far as the end of the ductus ejaculatorius. The observation that in *Cimex* the ducts are formed entirely from the loops themselves without the aid of any special outgrowth is supported by the condition in the adult, there being in this case no common duct as in some insects.

As regards the development of the external genitalia of the male in *Cimex* our observations therefore show that the conclusions originally based upon comparative studies are correct and that the asymmetrical arrangement of the parts is a special adaptation of the ordinary condition in the Heteroptera. It is of interest to observe in this case the appearance during development, of the right appendage, a structure which, if present at all in the adult, is not easily demonstrated.

The earlier stages in the development of the male organs appears to be in close accord with the findings of Zander<sup>(9)</sup>, <sup>(10)</sup> in the Hymenoptera and Trichoptera; and though the bug, on account of the small size of the organs, is not perhaps the most suitable form in which to study these early changes, yet our observations distinctly suggest that the *lateral appendages* in the Heteroptera are homologous with the *valvae* of Zander.

## PLATE XXXI

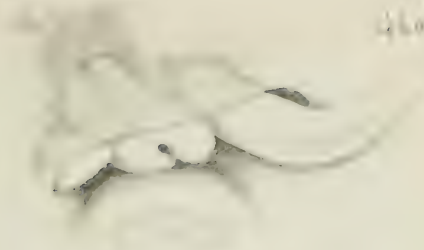
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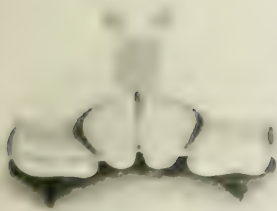
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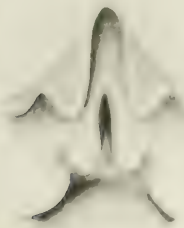
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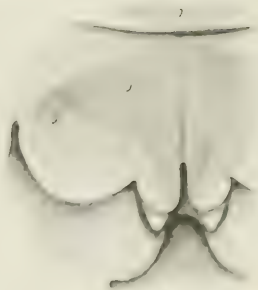
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4.



6.



7.



## CONCLUSIONS.

1. The abdominal segments in the bed-bug have not so far been correctly notated. The condition in the nymph and other considerations, including the presence of a relic of the first tergite, the position of the last pair of spiracles and the relations of the segments to the genital passages, etc., show that the large apparent first segment is the true second abdominal segment.

2. The female opening is behind the eighth sternite. The processes arising from this sternite are the *gonapophyses of the eighth segment* or *valvulae inferiores* of some authors. The peculiar lobes described by Rothschild are the *gonapophyses of the ninth segment*, or *valvulae intermediae* of some authors. The *valvulae superiores* (gonapods, styloids) though seen in some Heteroptera are absent in *Cimex*. The prominences on either side of the genital furrow are divided halves of the ninth sternite corresponding to the lateral chitinous portions of the sternal plate in the last instar nymph.

3. The male opening is behind the ninth sternite and between this and the tenth segment. The hollow in the ninth sternite (genital cavity) in which the male organs usually lie in the Heteroptera is reduced in *Cimex* to a tiny oval pouch enclosing the phallosome. The *phallosome* is a structure that has so far been overlooked. It lies at the root of the so-called *penis* and has all the chief characters of the phallosome in the order generally, though greatly reduced in size and complexity. The grooved false penis is one of the pair of processes (*lateral appendages* of Sharp) present with a few exceptions throughout the whole of the Heteroptera and higher Homoptera. The groove in the appendage does not function as a duct, but as a sheath for the mesosomal portion of the phallosome which can pass along it.

4. The sex can be distinguished exteriorly in the nymph, even when first hatched from the egg. The female parts are clearly indicated in the last instar of the female nymph and an asymmetrical chitinous complex is seen in the male nymph in the last instar behind the ninth sternal chitinisation, which represents the stage of development reached by the epidermal layer at the end of the penultimate nymphal instar.

5. The so-called penis is formed by the development of one of the bilaterally arranged processes which arise from division of the primitive projections. The outgrowth becoming the penis (the left appendage) continues to develop whilst that on the right eventually disappears.

The phallosome is formed from folds on either side of the early rudiment of the ejaculatory invagination and appears to be, as described by Zander for the Hymenoptera and Trichoptera, formed from the inner portions of the two primitive projections springing from the primitive genital cavity. It is at first an asymmetrical organ with a ventral groove. The stages by which it seemingly becomes a tubular organ require further study.

The vasa deferentia and accessory glands develop from a pair of small globular rudiments, one on either side, which arise independently of the ejaculatory invagination of the epidermal layer and are terminal dilatations of tubes which pass down, one on either side from the testes. The rudiments elongate and form on either side a U-shaped tube. The inner limb of each loop becomes the vas deferens, the outer the accessory gland complex. The bights of the loops grow downwards to meet the epidermal ejaculatory invagination, so that when eventually these open into this, two ducts and two separate organs, the vas deferens and the accessory gland, are formed on each side. The muscular fibres of the sacculus form round the bights of the loops as they grow to meet the epidermal invagination.

The ejaculatory duct is formed from an invagination of the epidermal layer which forms a spherical cavity with a button-like cellular plaque on its antero-ventral wall. The spherical cavity becomes the ejaculatory duct of the adult. The plaque becomes the *velum*, which projects into the ejaculatory duct and carries the openings of the four ducts derived from the bights of the loops described above.

6. The lateral appendages of the Heteroptera, so far as can be judged from our observations on *Cimex*, develop in the same manner as the *valvae* of Zander.

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## ‘ MELANOTIC GROWTHS.’

### ‘ RESEARCHES ON THE ALTERATIONS IN THE MELANOBLASTIC LAYER OF VERTEBRATES, TO EXPLAIN THE STRUCTURE AND ORIGIN OF THESE GROWTHS.’

BY

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[Received for publication, March 5, 1921.]

#### SECTION I.

THE nature and origin of melanotic tumours has given rise to a good deal of discussion, owing to the fact that the melanoblasts have not been investigated from the point of view of comparative histology. One is led to believe by reading most histology text-books, that in coloured races the pigment can be formed by the columnar epithelial cells. Wilmot Evans (1901), Sampson-Handley (1908) and others consider that melanin is produced by specialized cells called chromatophores or melanoblasts. Sufficient evidence was not brought forward to make the latter view generally acceptable.

The structure of benign growths (moles) was little studied, until Unna challenged Von Recklinghausen's view (1908) on the endothelial origin of the naevoid cells of soft moles. Since Unna published his paper, controversy has raged between the two schools (Whitfield 1905, Wilfield Fox 1906, Johnson 1905, and others). The literature shows that the dual nature of the cells in these growths (*i.e.*, melanoblasts and angioblasts) was not sufficiently realised. The nature of the malignant melanomata is in a still greater state of confusion, these growths are variously regarded as sarcomata, carcinomata, or epitheliomata, according to the opinions held by different investigators.

In 1904, I commenced a study on these neoplasms and found that in moles, two types of cells were constantly present, *viz.*—

(i) Melanoblasts, or pigment cells of Deiter. The term melanoblast will be used throughout this paper, instead of chromatophores for reasons that will be evident later on.

(ii) Angeioblasts, or nævoid cells.

From this fact, I surmised that two different types of malignant neoplasms could arise from the one benign tumour (pigmented mole):—

(a) A spindle-celled melanotic sarcoma originating from the melanoblasts.

(b) An endothelioma from the angeioblasts.

In a previous paper 1905, I could not obtain sufficient evidence to make this view tenable:—

(a) because I was unable to account for the presence of embryonic melanoblasts and angeioblasts lying side by side in these pigmented moles;

(b) neither could I exclude the possibility of a pigmented growth arising from the columnar cells of the epidermis;

(c) nor could I interpret the blending of the cells in malignant melanomata, when arising from moles.

At this incomplete stage my paper was published. Sir John Bland Sutton, in his preface 'On tumours innocent and malignant,' gives the gist of his teaching of tumour pathology by stating 'whenever it seemed advisable to illustrate the nature of a genus of tumours by comparative pathology I have not hesitated to do so. Without its aid any attempt to catch the deeper meaning of many tumours is as difficult as endeavours to decipher a palimpsest in which the first characters, written in an unknown tongue, have been imperfectly removed from the parchment and are allowed to mingle with the second inscription.'

With the aid of comparative morphology, the entire subject of this investigation became clear, for it seemed as if the whole story of the melanoblasts had been indelibly written in the dermis of vertebrates. In many of the fishes, amphibians, and reptiles, the melanoblasts still retain their full development and primitive functions, and form a more or less complete pigment sheet. With the appearance of dermal appendages, this sheet becomes associated with the appendage in particular, so that in lower mammalia we find the melanoblasts chiefly concerned with the pigmentation of hairs. In higher anthropoids, a gradual loss of the hairy coat occurs, and in the highest anthropoid,

man, we find the hairy coat has almost disappeared, that the melanoblasts of the general body surface are no longer applied to the hair roots and have lost their function of pigmentation. When this loss of function occurs, atrophy sets in. In coloured races these cells are greatly altered in shape and size, and are recognized with difficulty, unless the changes in lower animals have been studied. They still retain the function of protecting the individual from the effects of solar rays, by virtue of their contained pigment. In white races, on the other hand, loss of function has gone a stage further, so that these cells, now unpigmented, cannot be recognised in the dermis, unless stimulated into activity by the sun's rays, *e.g.*, freckles and bronzing of the skin. Relics of the sheet are still to be seen in various positions of the body, *e.g.*, the choroid and genital area; and appear vestigially in the form of pigmented moles. A study of the pigment vascular layer of amphibians shows the close connection between melanoblasts and angioblasts, an important point in the study of melanotic growths.

In 1905, records were collected of over 200 cases of malignant melanomata and emphasis was laid on the importance of removing the growth with the whole of its lymphatic area. In every case in which details of treatment were recorded, the growth was removed, whilst the permeated lymphatics and glands were invariably left alone. As excision was not usually performed wide of the growth, the permeated lymphatics were cut across and their contents strewn all over the wound, so that numerous secondary nodules appeared in the vicinity of the scar. Mr. Sampson-Handley has thoroughly worked out the mode of dissemination of these neoplasms and has pointed out the technique to be employed in their removal.

In this paper I will give my views on the structure of this group of neoplasms, by treating the subject in three sections. The first section will deal with the *Melanoblasts* giving the result of my studies, on their structure, development, arrangement, as well as their relation to the dermal appendages. At the same time, facts will be brought forward to show how they are the only cells concerned with melanin production. The second section will deal with the origin of *Benign melanomata* (moles) from suppressed hair fields, and from this we can interpret the structure of these growths. The third section will be concerned with the structure of *malignant melanomata*.



## PLATE XXXII.

Section of the skin of a shark showing dermic hooklets and under them are large branching melanoblasts which are diffusing melanin into the surrounding tissues.

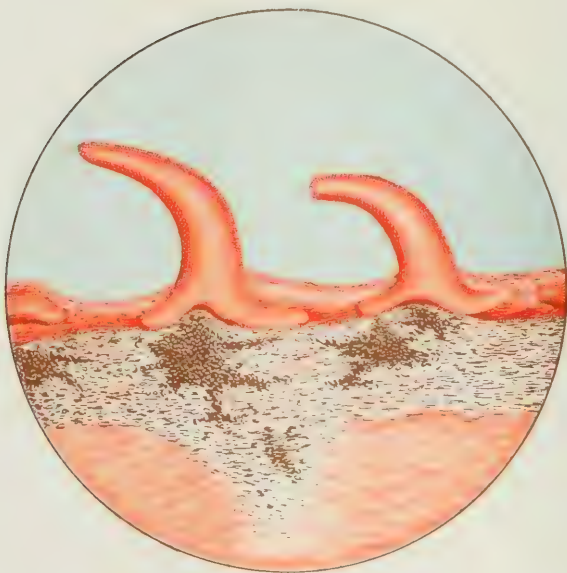




PLATE XXXIII.



A pigmented mole arising in contraction with the  
fronto-maxillary cleft.

## MELANOBLASTS.

These cells in lower animals (Reptiles, Amphibians, etc.) are stellate in shape with long dendritic processes and deeply pigmented with melanin. The granules, although obscuring the nucleus from view, sharply demarcate the cell body and processes causing an appearance as if the cell had been stained by Golgi's method. The protoplasmic processes are extremely sensitive to light, immediately contracting on exposure and concentrating the pigment in the cell. At rest, the melanoblasts (see Plate XXXII) are seen lying passively with their processes fully extended and round them are numerous tiny granules of melanin, that are leaving the cells and drifting towards the epidermis, to permeate the intercellular channels between the columnar cells. This diffusion of pigment I wish to emphasise, as it explains why melanin was thought to be elaborated by the columnar cells of the epidermis. It also shows how the cells during their periods of rest elaborate pigment to form a colour filter in the epidermis, so as to protect the sensitive dermic structures which are lying under it. Further, it accounts for the situation of the pigment in both simple and malignant melanomata; for, if diffusion takes place to any extent, the angioblasts and plasma cells, owing to their phagocytic properties, will certainly contain melanin. We also learn from the effects of light how the cells of internal growths are deeply pigmented. In these situations not only is their nourishment more abundant, but the oxidizing effects of light are practically absent. The above description applies to the fully developed melanoblasts, but from a study of their embryonic life (see Plate L) they arise as unpigmented cells of the mesoblast and develop pigment later. As development proceeds they become elongated, fusiform in shape, and their secretion is recognized by the production of minute yellow granules. Processes in the shape of a long pennant grow out from either pole of the cell. The pigment increases in amount and depth, finally the processes become dendritic and the cells reach maturity. This method of development is further borne out by studying the spindle cells in malignant melanomata. The malignant spindle cells when young and rapidly dividing are unpigmented, fusiform in shape, and unbranched. At this stage, according to Adams's view, their secretion is in abeyance. Soon some of the cells become elongated, bipolar in shape, and melanin granules are formed. In these older cells I have observed somatic division, indicating a possible tendency toward a more normal method of cell division.

In malignant melanomata, I have never observed development of the spindle cell to proceed to its fullest extent, for even the most mature cells do not advance further than the bipolar stage.

#### DEVELOPMENT OF THE MELANOBLASTS.

The most convenient situation to observe the development of the melanoblasts is the hair. In man, the lanugo hair begins to form in the fifth month of intra-uterine life as solid buds from the rete mucosum and basal layers of the epidermis. As these buds grow down into the mesoblast, they compress it, and at the point of compression, the mesoblast reacts to form a vascular papilla. This papilla invaginates the epiblast and prevents any further inroad into the mesoblast. My observations show that concurrently with this epiblastic invasion, ovoid cells make their appearance along the edge of the mesoblastic papilla and lie apart from the cells of the invaginated epithelial bud. These are embryonic melanoblasts; at first they have vesicular nuclei indicative of their rapid division: soon the ovoid shape is lost, the cell becomes fusiform, processes grow out, and melanin is secreted.

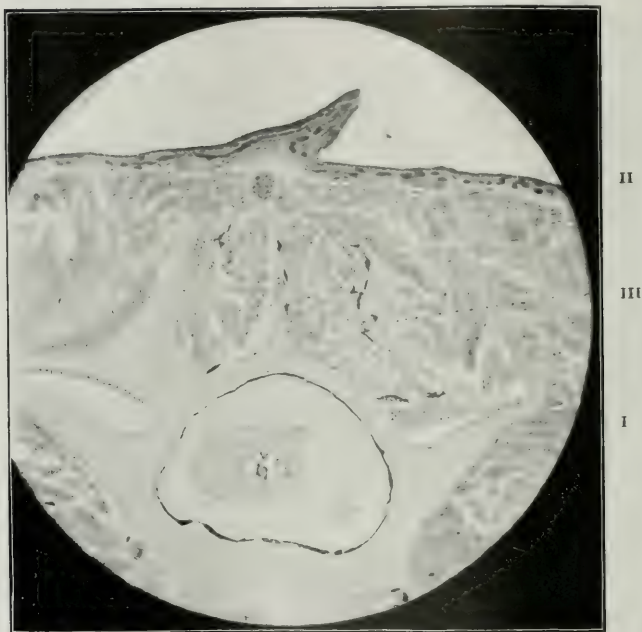
Synchronously with these changes in shape, etc., a gradual alteration is seen to take place in the position of these cells, until finally they insinuate themselves between the cells of the inner hair sheath (basal layer of the epidermis). As the melanin is formed and diffuses into the intercellular spaces, a pigmented mass is produced, which lies next to the vascular papilla. Any microscopic differentiation of the cellular constituents of this mass is now no longer possible.

The same process was studied in the foot p d of a kitten embryo (see Plate L). Here the melanoblasts are quite distinct and gradually insinuate themselves between the columnar cells, but their structure can always be made out with ease in comparison with those in the dermis of coloured races. So far I have regarded these cells as being situated only in the dermis, so will have to explain the presence of melanoblasts in the mucous membranes of the buccal and genital areas, and pia-arachnoid. This point may best be illustrated by a study of sections of the skin of amphibian embryos, and for convenience that of the *Rana esculenta* was chosen. A large, well-developed pigment layer is seen lying under the epiblast and closely connected with the superficial respiratory blood vessels. To this layer I have applied the name 'primitive pigment vascular sheet' as from it I believe that all the melanoblasts of the body are ultimately derived. As the epiblastic



PLATE XXXIV.

No 4. 3rd objective.



Section through the body of a newt.

- (I) Showing the Pigmentation of the pia-arachnoid.
- (II) Melanoblasts of the Corium.
- (III) Melanoblasts which have persisted after being carried down by the neural canal.



PLATE XXXV.

No. 4. Eyepiece 3rd objective.



Section through the glans penis of a human foetus, showing the formation of the prepuce.

Note—The melanoblasts around the epithelial process, and their concentration at the head of the epithelial bud.

invagination which is going to form the central nervous system sinks down into the mesoblast, it is gradually surrounded by a portion of the 'primitive pigment vascular sheet,' which gives rise to the pigmented pia-arachnoid of amphibians (see Plate XXXIV). In man the vascular elements of this sheet alone persist, and form the endothelial lining of the vascular spaces of this membrane. The choroid may be in the same way derived from the pigmented pia-arachnoid. As regards the buccal and genital mucous membranes I found the same process of invagination from the general surface to take place (see Plate XXXV), and so the presence of melanoblasts in these situations was similarly explained. Invaginations also occur at the gillefts in amphibians.

#### FUNCTIONS OF THE MELANOBLASTS.

In fishes, amphibians and reptiles these cells are highly developed and retain their full functions, *viz.* :—

- (i) Protection, as illustrated by alteration of colour with surroundings, mimicry, etc.
- (ii) Differentiation of species according to the arrangement and character of colouration.
- (iii) Differentiation of sex. In some species, during the breeding season, secondary sexual characteristics are acquired by the increase in amount of the pigment.

From my observations it appears that as soon as the dermal appendages make their appearance in the skin, the melanoblasts become closely applied to them so that their functions become almost identical. The hair being the most interesting and important of these dermal appendages and a characteristic feature of all mammals, a brief study of its Phylogeny will throw further light on the view advanced.

The mailed amphibians (*Stegocephala*) of the carboniferous strata possessed a complete coat of long scales which were derived from the corium and over this was a covering of bony scales. On passing from aquatic to terrestrial life, the dermic scales became more fully developed and the bony scales degenerated, this is seen in the fossil reptiles (*Palaeohatteria*, *Homocœsaurus*, etc.), which are found in the upper Permian strata. As regards the feathers of birds, it is nearly certain that they are modifications of the horny scales, but this is not the case with the hair of mammals. Maurer, after extensive researches, showed :— that as the conical epidermal buds of the skin-sense layer of amphibians came to project from the general skin

surface, they did so under cover of the horny layer. This horny layer in turn increased in size by cornification, until the whole structure (dermal appendage, and nerve endings) became an end organ of sensation, *e.g.*, tactile hairs. In many mammals, these hairs are still sensory, *e.g.*, the tactile hairs on the muzzle and cheeks of cats and dogs. Maurer also pointed out, that the scales and cutaneous sense organs were arranged in regular longitudinal ridges in the lower mammalia, whilst the hairs in higher mammalia were similarly arranged.

A study of the embryo rat, and other embryos, shows that hairs are originally arranged in regular longitudinal ridges, to which I have applied the name 'primary hair ridges.'

These primary hair ridges run transversely to the longitudinal axis of the body; and as growth takes place those near the head and tail end tend to diverge from parallelism. Soon these ridges break up into serial areas 'hair fields.' When this occurs, the hair fields appear to be arranged in transverse, longitudinal, or oblique lines according to the position of the observer (see Plate XXXVI).

The arrangement of these ridges explains the transverse stripe markings in many animals, *e.g.*, the tiger and zebra, whilst evidences of the hair fields are shown in the spot markings in some of the carnivora, *e.g.*, the leopard, and the longitudinal stripe markings in some of the rodents, *e.g.*, the badger and squirrel.

Between and in these hair fields, the small scattered lanugo hair are seen arising from the 'primary hair follicles.' The irregular pigmentation of small or large areas of primary hair follicles shows how mottling or piebald patches occur in various mammals. This arrangement of the hair ridges and fields explains the mode of origin of the *nævus linearis* and *N. unius lateralis*, as well as the large moles so frequently seen on the back and chest. The close connection of nerve areas to giant *nævi* is likewise explained.

#### RETROGRESSION OF MELANOBLASTS.

Most of the animals of the mammalian kingdom are provided with a well-developed hairy coat. In the higher catarrhine apes, a marked diminution of this hairy coat is seen on various parts of the body. The particular process by which man lost the greater part of his hairy coat, and in other parts of the body retained or even augmented it, is, according to Darwin, due to sexual selection. At one time it was disputed whether melanoblasts occurred in the dermis of man, for it

PLATE XXXVI.



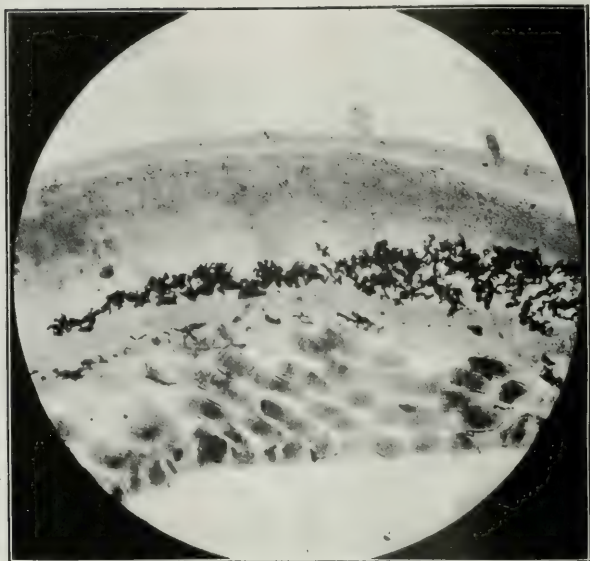
A bear embryo (after Haeckel), showing the arrangement  
of the primary hair ridges and hair fields





PLATE XXXVII.

No. 4. Eyepiece  $\frac{2}{3}$ rd objective.



Section of the skin of a Russell's Viper, through a spot marking.  
Note—The aggregation of the melanoblast under the corium.



PLATE XXXVIII.

No. 4. Eyepiece  $\frac{1}{4}$ th objective.



Melanoblasts in the corium of a newt's skin.

Note—Their large size and irregular branching processes.

appeared that the columnar cells had usurped this function, and that the melanoblasts had completely disappeared. But a comparative morphological study of these cells shows that, as the dermal appendages made their appearance, there has been a gradual migration of the melanoblasts towards the epidermis and a retrogression in their shape, size and function, concurrently with the loss of hair from the general surface. To illustrate this view reference will be made to the following vertebrates, which have been studied.

- (I) Reptiles. Sections of the skin were examined from many of the lizards and snakes found in this country and as a type example that of the *Viper russelli* will be described. In the corium of this snake, large well-developed melanoblasts are seen lying some distance away from the epidermal scales; and under the chain markings, they are seen aggregated in large clumps (see Plate XXXVII), and often near the small blood vessels of the corium (see Plate XXXVIII). This may be regarded as the first stage in their history, when they are lying some distance from the epidermal scales.
- (II) Fishes. In the skin of dogfish and shark are seen large branching melanoblasts, which contain numerous tiny granules of a dirty brown pigment. These cells are situated at the base of the dermic hooklets (see Plate XXXII) and are in close apposition to the numerous small capillaries which go towards the hooklets. This stage in their history illustrates the earliest tendency of these melanoblasts to approximate themselves to the dermal appendages.
- (III) In amphibians the melanoblasts are closely connected with the cutaneous-respiratory plexus, and are large in size, fully developed, lying quite apart from the epidermis and extend some way into the mesoblast. The epidermis is often deeply pigmented over those spots, where the melanoblasts aggregate in clumps in the cutis vera. The pigmentation of the epidermal cells is produced by the permeation of free melanin into the intercellular lymph-channels. Melanoblasts also occur in the pia-mater, and buccal mucous membrane of these animals, and here again they lie in proximity to blood vessels. This close vascular connection is important to note when studying the nature

of simple and malignant melanomata. In these three types of animals, the melanoblasts are well developed, often lie quite apart from the epidermis, and are situated near blood vessels.

(IV) Mammalia.

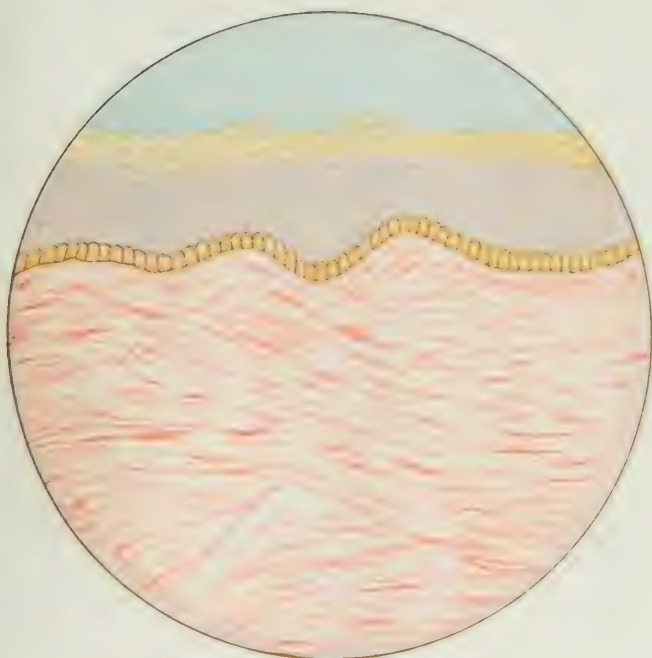
(A) Sections were made from the skin of the general surface and from the special pigmented regions (*e.g.*, snout and genitalia) of the lower mammals. The latter areas are almost devoid of hair, the melanoblasts are smaller, ill developed, and approach the epidermis more closely. Their recognition becomes more difficult than in the three preceding groups. Sections of the hairy coat show that the melanoblasts have for the most part left the general skin-surface layer and are solely concerned with the pigmentation of the hair; when they are recognised, they are seen to be closely applied to the columnar cells of the epidermis.

(B) Man.—The choroid is the only situation where the melanoblasts still retain their primitive type, for here they are large, branching, deeply pigmented, retain the power of contracting to light, and have the close relationship to the blood vessels. In the genital area, they occur as scattered fusiform cells containing brownish granules, and insinuate their processes between the columnar cells, so that their shape is almost unrecognisable. They are best observed during the height of sexual life. The buccal mucous membrane in man is as a rule unpigmented, but in natives of India (those of Dravidian origin) I have frequently noticed pigment patches on the inner surface of the cheek and tongue.

In the epidermis of coloured races, the columnar cells appear to be pigmented (see Plate XXXIX). The appearance is due to delicate pigmented processes belonging to melanoblasts. The bodies of these melanoblasts are very slender, their processes very fine and completely surround the columnar cells in a similar manner to the neuroglial cells which embrace the Purkinji cells of the cerebellum. Owing to the diffusion of pigment, there is a brownish haze over the columnar cells. Therefore recognition of the melanoblasts in coloured races, as a separate group of cells, is extremely difficult, until one has studied their history and seen how the alteration in shape, size, and situation have occurred.

I regard this dermic migration of the melanoblasts as a compensatory change, in order to protect a large skin surface, these cells have had to migrate up towards the epidermis, become thinner, more elongated and branched. In white races, the compensation has

PLATE XXXIX.



Section of the skin of a Negro showing the apparent diffuse pigmentation on the columnar cells.



not occurred owing to climatic and adaptive conditions, with the result that exposure to strong sunlight is soon followed by violent vascular changes, *e.g.*, sun-burns. It is not until the process of tanning has been endured that protection from the sun's rays is established, or, put in other words:—In white races protection from the sun's rays is not acquired until the potential melanoblasts are stimulated into activity, and, by elaborating melanin form a yellow filter to obstruct the heat rays from injuring the vessels in the underlying dermis.

#### MELANIN.

As melanin is such a characteristic feature of the melanoblasts, it is necessary to note the following facts, in order to eliminate errors that may occur from the presence of other pigments, *viz.*, blood pigments, etc., that may be ingested by the tissue cells.

Melanin occurs in the form of fine irregular granules or acicular particles varying in colour from a golden brown to an intense black. Chemically it contains a large proportion of sulphur (*i.e.*, 8 to 13 per cent), no iron is present. The chemical properties of melanin, and the test for iron are well known and will not be discussed.

Schmidt contested the point as to the absence of iron, and has shown that iron-free pigments can be obtained experimentally from hæmoglobin; he has also observed the presence of iron-containing pigments in the cells of malignant melanomata, but noted that the bulk of the pigment present was iron free. From these facts he concluded that melanin was derived from blood pigments and in the earliest stage of its formation must contain iron. The iron was eliminated by cellular activity from the chemical composition of the blood pigment and so was transformed into melanin. These neoplasms are very vascular and as the vessels are mere blood spaces, they readily allow extravasations to occur, sometimes to such an extent that the bulk of the colour of the primary growths is largely due to these hæmorrhages.

It is only within recent years, that Sarcoma idiopathicum multiplex pigmentosum cutis (Kaposi's type) has been separated clinically from the melanomata, for, in the former, the cells are filled with blood pigment and not melanin. In the melanoblasts of the dermis, and in those that occur in moles, I have never observed the presence of an iron-containing pigment. In these situations, the vessel walls are normal, and hence no complicating blood extravasation occurs. In malignant melanomata a different state of affairs exists, and I have frequently observed

iron-containing pigments in the angioblasts and plasma cells, and very occasionally in the melanoblasts.

In consideration of these facts I conclude that any iron-containing pigment is not melanin or even a precursor of it.

#### ORIGIN OF MELANIN.

There can be little doubt that the melanoblasts in lower animals form melanin in their cell body (see the researches of Erhmann, 1887, and Nothnagel). My study, 1905, of the developmental changes in these cells has shown that the pigment network between the columnar cells consists really of the processes of melanoblasts (the researches of Mr. Sampson-Handley, 1908, and Wilmot Evans, 1901, confirm this view); therefore we undoubtedly have in coloured races retrogressed melanoblasts that form pigment in the cell body just as in the case of lower animals. Sufficient evidence must be brought forward to prove that the columnar epidermal cells play no part in forming melanin. Karg pointed out that if the skin of a white man was grafted on to a negro it became pigmented, whilst if the converse was done, the dark skin rapidly became depigmented. From this experiment, we are led to believe that the dermis is the seat of formation of this pigment, the epithelial layer being only passively impregnated from the dermis. This view of Karg's is confirmed in a study of the effects of burns of the skin in coloured races. A patient was under my care in Peshawar suffering from an extensive burn of the third degree which was situated on his back. The epithelium grew over the area some 18 inches square, and for two months the scar remained white, it then gradually became pigmented by an extension of the pigment from the edge. When last seen (*i.e.*, 18 months after being discharged from the hospital) the scar was still white in the centre, indicating that although the epithelium had been fully formed pigmentation had to take place from the edge of the scar by an ingrowth of the melanoblasts. In burns of the second degree on the other hand, the involved area is lighter in colour, but in a short time becomes rapidly and evenly pigmented, showing that the vital elements have not been destroyed. Therefore, I conclude that melanin must be formed in the melanoblasts alone. It may be produced in the following ways :

- (1) as a secretory process, the result of cellular activity, or
- (2) as the result of ingestion of blood pigment which is then converted into melanin.

The latter theory can readily be dismissed, for we could not explain the depigmentation of the skin of white races and albinos as they have the same blood pigments and vessels as coloured races. Albinism on the other hand, is explained by the theory of retrogressive changes in the melanoblasts whereby these cells lose their function wholly or partly. Furthermore, if the production of melanin was of the nature of a transformation of blood pigment, we should expect to find all gradations from a pigment-like haemosiderin up to melanin; this has never been demonstrated. To some observers the close connection of melanoblasts to the blood vessels has served as an important point in upholding the view that melanin is formed from haemosiderin or some allied blood pigment, but in many studies show that many of the melanoblasts have been divorced from this close vascular relationship, but still continue to form melanin. Thus we may reasonably postulate that melanin is formed in the body of specific cells (melanoblasts) and is not derived from blood pigment. Rossle, Meirrowsky, and Staffel have a different view on the origin of melanin, regarding it as a derivative from the nucleolar matter of the melanoblasts, and look upon its presence as a distinct sign of nuclear exhaustion or degeneration; this view is not borne out by a study of the normal melanoblasts, for the most mature virile cells contain the largest amount of pigment. In the young melanoblasts of malignant melanomata where division is rapid, there is little or no melanin, it is only in the mature cells of these tumours that much melanin is developed. In other words it is not until secretion is established that melanin is seen. von Firth advanced the theory that melanin is formed from the proteid molecule of the cell by the action of an enzyme (tyrosinase). He has further shown that an enzyme (tyrosinase) is present in the ink sacs of cuttle-fish, and develops the pigment sepia, which is closely allied to melanin. Following von Firth we may explain the bronzing of the skin in this disease as due to the action of the tyrosinase of the melanoblasts which converts tyrosin into melanin.

This idea is further borne out by the development of the melanoblasts of the dermis, and in those of malignant melanomata. In the stage of rapid division, where the cells are chiefly vegetative, they contain no melanin, i.e., the secretory function is in abeyance. As growth becomes less rapid this function comes into play, and melanin is formed. Meirrowsky subjected small areas of skin in Europeans to the action of Finsen light and saw that pigment accumulated in these white skins more particularly on the side nearest the light, he

observed these cells to send out processes between the columnar cells, and even into the cutis and assume the characteristic melanoblastic type. This experiment shows that there exists, in the dermis of fair races, cells which do not secrete pigment, unless when stimulated into activity by actinic rays. Melanoblasts exist in the ora serrata of albino rabbits without any melanin in their cell and body, showing that the absence of pigmentation is due to a deficiency in secretion, and not to the absence of these cells.

The absence of melanin can be explained as due to an inherited quality of the cell whereby the nucleolus has lost its secretory power of producing tyrosinase. I conclude, therefore, in favour of a secretory origin of melanin and that it is very probably formed through the action of an enzyme derived from the nucleolus.

#### FATE OF MELANIN.

There is always a tendency for the pigment to diffuse from its seat of production, travel up towards the epidermis, and permeate the intercellular spaces between the columnar cells. Higher up, that is, in the prickle cell layer, there is hardly a trace of this pigment to be seen, indicating that a change has taken place in the melanin. Miura first pointed out that the granules seen in the nævoid and plasma cells, and even in the leucocytes of connective tissue, were altered in colour suggesting an oxydation of melanin. The melanin seen in the angioblasts of innocent melano-endotheliomata (moles) invariably appears to be a lighter coloured pigment, as if it was being transformed into an achromatic substance. In rapidly growing moles overproduction of melanin sometimes occurs and the tissue cells are unable to deal with it locally; leucocytes can be seen carrying melanin granules to the lymphatic glands. In malignant melanomata, melanin granules have been observed in the plasma and even in the leucocytes of the circulating blood (Nepvin). Sometimes the condition may be so marked so as to cause a general pigmentation of the face (melanæmia) and to be excreted by the urine (melanuria). A commoner condition is met with in the final stages of these malignant growths, the melanin is partly destroyed and excreted in the urine as an achromatic substance that turns black on exposure to air or by reagents as Bromine water. von Jaksch has given the name melanogen (to this substance) as he supposes it to be a precursor of melanin. The melanogen only appears late in the disease, and very

frequently may not be present. Sufficient evidence is not forthcoming to label this achromatic substance a precursor of melanin. It may just as likely be a reduction product.

## SECTION II.

### *Benign Melanomata.*

In the previous section I pointed out, that whenever any inroad of epiblast occurred into the mesoblast, it carried before it, a portion of the 'primitive pigment vascular sheet.' In the development of the hair we saw how the mesoblastic papilla (consisting of angioblasts and melanoblasts) met the epidermal bud, and prevented any further down-growth of it into the mesoblast. Should suppression of growth of the epithelial bud take place, the mesoblastic papilla may persist and give rise to a mole. If this view is correct, these moles should only be in situations where epidermal downgrowths occur.

On the face, moles are commonly seen in the line of the various facial clefts, they have been described in connection with the Fronto-maxillary cleft (Plate XXXIII), and at the Naso-facial cleft, and also at the point of union of the mandibular processes. I saw a very interesting example in a Native of India of a mole arising in the position of the branchial cleft. A narrow strip of melanomatous tissue  $\frac{1}{4}$ " broad and 1" long extended from over the great cornu of the hyoid to below the angle of the jaw corresponding in position to the second branchial cleft. The middle line of the body is also a common situation, especially near the supra-sternal notch. When examining men for enlistment, I saw numerous examples of moles rising from the primary mammary ridges. Although these situations account for a large number of the melanomata seen, yet their occurrence on other parts of the body requires further explanation. Maurer pointed out, how the hair first arises in connection with the primitive sense organs of amphibians, and from this we see how nerve areas or groups of nerve areas become associated with melanomatous patches, (e.g., giant naevi. I have already discussed, how the primitive pigment sheet is broken up into longitudinal and transverse ridges, the suppression of which accounts for the *N. linearis* and *unius lateralis*; and how the primary hair ridges are, in their turn, split up into hair heads. If both the epiblastic and mesoblastic elements of these fields persist it would account for the *Melanopapishoma*

(Hard Nævus). If development proceed slightly further, *i.e.*, to the formation of hair, the Nævus pilaris will be formed, whilst if only the mesoblastic papilla persists and atrophy of the epiblastic downgrowth occurs, it would account for the large bald moles seen on the back, abdomen, and chest. The small moles scattered over various parts of the body, probably have their origin in the suppression of the primary follicles of the lanugo hair. Moles in any situation on the body can therefore be explained as due to some irregularity in the development of the epiblastic invagination. I will now proceed to the consideration of melanotic growths.

#### INNOCENT MELANOMATA.

The term signifies any circumscribed pigmented area of skin or mucous membrane which is congenital in origin and pigmented by melanin. This definition will include the following clinical varieties which have been reclassified as the result of my histological findings.

(A) Benign melanomata are pigmented areas of skin, mesoblastic in origin, and composed of melanoblasts, they represent the pigment patches seen in the skin of lower vertebrates.

The following are the clinical varieties described :—

(I) *M. solaris* (freckles).

(II) *M. spilus*.

(III) *M. cœruleus* of Max Tièche.

(B) Benign melano-epitheliomata or hard Nævi. These are areas chiefly epiblastic in origin, and composed of a hypertrophy of the prickle cell layer, containing melanoblasts in the corium. I regard them as suppressed hair fields, where the epiblastic element has mainly persisted.

(C) Benign melano-endotheliomata. I previously pointed out that should suppression of the epiblastic downgrowth occur the elements of the reacting mesoblast may also persist, so that there is a mesoblastic papilla consisting of melanoblasts and angioblasts, lying side by side. This congenital anomaly of the skin is known as a pigmented mole. The following are the clinical varieties recognised :

(i) Melano-endothelioma gigas.

(ii) M-endothelioma linearis, including the subvariety M-endothelioma unius lateralis.

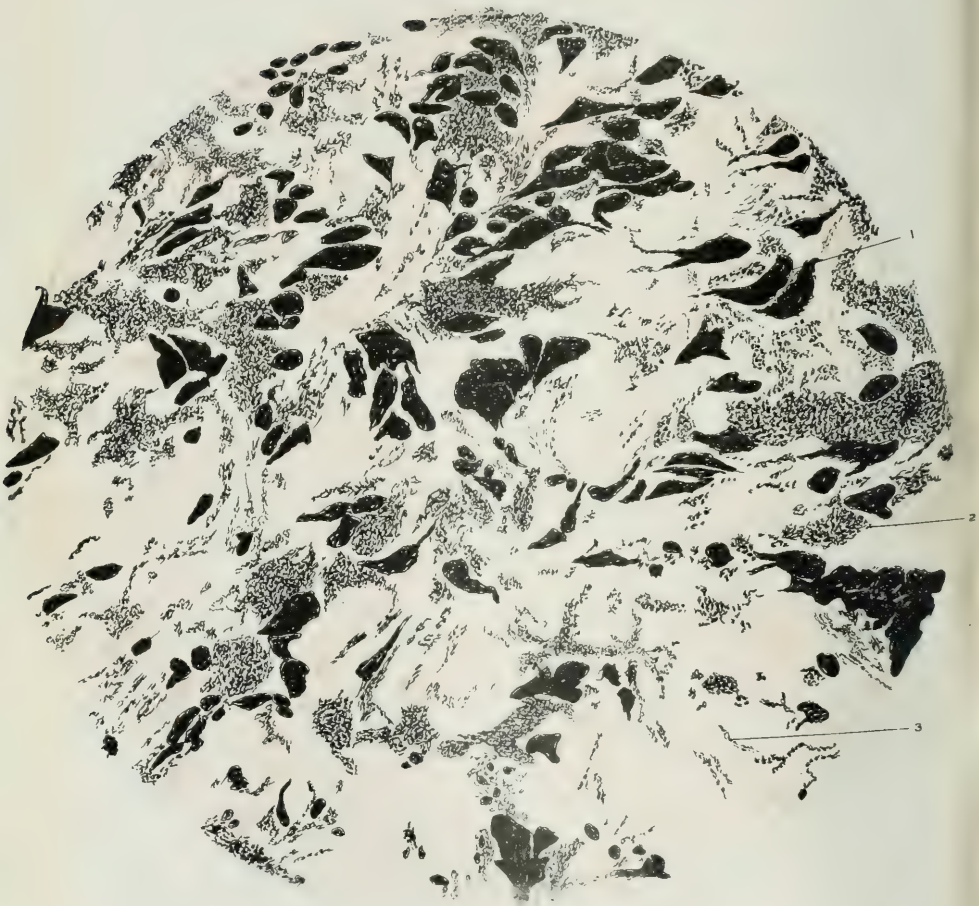
(iii) M-endothelioma mollusciformis.



## PLATE XL.

Section of a benign melanoma showing

- (1) Mature melanoblasts
- (2) Young melanoblasts
- (3) Processes and masses of melanin.



(re) M-endothelioma papillomatosus

(c) M-endothelioma pilaris.

(D) Benign endotheliomata or unpigmented Moles. These have been included in the classification, as in my opinion they have the same origin as the preceding (C), but differ in the fact, that the melanoblasts have not developed in sufficient numbers to effect pigmentation. The growth therefore consists solely of angioblast. A brief clinical description of these innocent melanomata with an account of their microscopic structure will be given before describing the nature of their malignant representatives.

(A) Simple melanomata.

(I) Freckles are small circumscribed patches of pigment, seen on the face, and hands, appearing usually in the second decade of life and are caused by the stimulation of potential melanoblasts by the sun's rays. The number and depth of colour varies inversely with the amount of exposure to sunlight, being most marked in the summer months, and almost disappearing in the winter.

(II) Melanoma spilus (Plate XLIII) is a flat area of pigmentation, occurring on the trunk and extremities (about the wrist and ankles) and consists of local aggregations of melanoblasts, probably similar to the pigment patches of reptiles and amphibians.

(III) Gunpowder Naevus of Max Tieche is blue in colour, like the gunpowder tatoo marks or the pigmented scars of miners.

The structure of these Benign melanomata, consist of melanoblasts only. The development of the melanoblasts varies in the three clinical forms being least developed in the freckle, and most highly developed in the Max Tieche Naevus. The latter has been carefully investigated and described by Dr. Max Tieche and, owing to Mr. Sampson-Handley's kindness, I was able to examine sections sent to him by Dr. Tieche. The cells seen were large, branching and perfectly black with pigment and lying some way from the epidermis reminding one of the melanoblasts seen in reptilia (see Plate XL).

(B) Melano-epitheliomata. Here the term Epithelioma is used in the continental sense; they form congenital localized growths of a part or whole of the epidermis and cap a large vascular papilla which is often pigmented. Dermatologists have subdivided these growths

histologically into three sub-groups, depending on the layers of the epidermis involved, viz.:—

- (i) Keratoid Nævus in which the stratum corneum alone is involved.
- (ii) Acanthoid Nævus in which the prickle cells are chiefly affected. (See Plate XLI).
- (iii) Mixed type of (i) and (ii.)

The Acanthoid type is the variety usually seen and this alone requires description. The prickle cell layer is enormously increased, the cells are smaller than normal, and the intercellular bridges are still well marked. Epithelial pearl-like masses are seen scattered on the surface, and among the prickle cells. The basal layer is usually indistinct, and often the prickle cells appear to end abruptly in the mesoblast. Long tongue-like processes of mesoblast extend into the papillæ and on oblique section appear as round masses of connective tissue among the prickle cells, as if they had been isolated from the corium. These papillæ are composed of numerous blood vessels and the melanoblasts are ill developed and lie close to the epidermis. Melanin pigment is seen diffusing into the epidermis. No medullary substance is visible as occurs in cutaneous horns. The hypertrophy of the epidermis associated with large mesoblastic pigmented papillæ suggests the possible origin of these nævi from a primary hair field. The baldness suggests suppression of development. The hypertrophy of the prickle cell layer shows that the epiblastic element of the hair field preponderates in this form of growth.

(C) Benign melano-endotheliomata.

- (i) Giant Nævi are large soft moles which occur on the lower part of the abdomen, buttocks, and thighs, unilateral or bilateral, giving rise to the bathing drawers pattern, and corresponding in position to the distribution of cutaneous nerves, or groups of nerves. A case of this clinical variety of mole was seen in a girl aged 14, who had a very extensive mole of the scalp, where the temporal and occipital nerve areas were chiefly involved.
- (ii) Linear Nævi are long strips of melanomatous tissue sometimes running the whole length of the limb. Their origin as I pointed out is connected with the primitive hair ridges. Various other hypotheses have been put forward, notably by von Baresprung, who suggested that they may



Large verrucose mole of the scalp.  
Note—The baldness of the mole.



Mole arising from the transverse and oblique hair ridges.

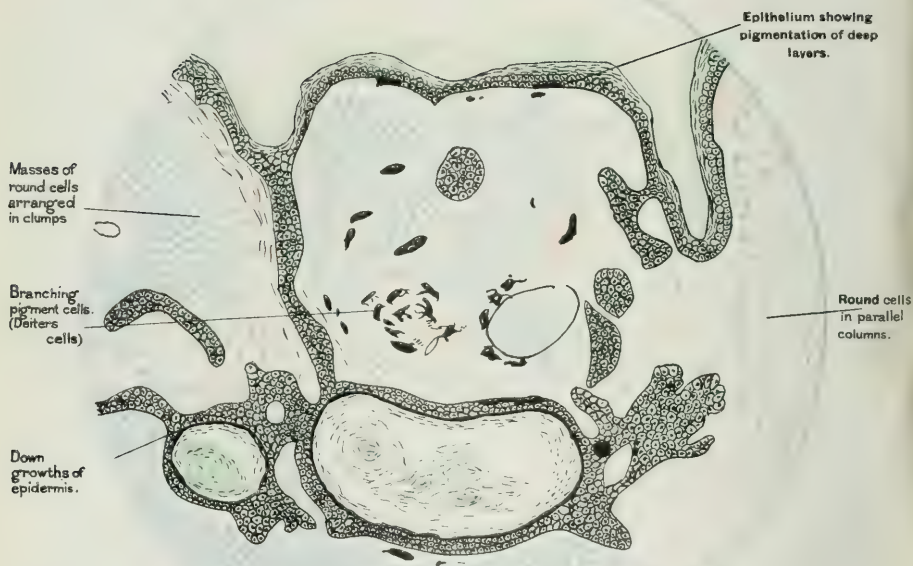
HUGH W. ACSTON, M.D.—'Melanotic Growths.'





## PLATE XLII.

Section of papillomatous mole  
(Benign M. endothelioma)  
Methylene blue and eosin.



be caused by some intra-uterine affection of the spinal ganglia producing a condition similar in distribution to Herpes Zoster. Phillipson thought that they may have some connection with Voigt's lines. The subvariety of this group, the *Nævus unius lateralis*, first described by Unna and von Baresprung, is simply a zosteriform type of the above and can be regarded as arising from the transverse hair ridges on the chest. (See Plate XLII).

- (iii) Mollusciform *Nævus* is simply a soft mole in which fibrosis has occurred at the base, the vascular and lymphatic supply obliterated and caused œdema. Unna found in the microscopical examination of 12 cases, 9 of them showed degenerated angioblasts.
- (iv) Papillomatous *Nævi* form a subvariety of these soft moles in which the epithelium proliferates in a papillary manner.
- (v) *Nævus pilaris* is a large mole, very dark in colour, and covered with lanugo-like hairs, corresponding to a partially aborted primary hair field in which some development of the primary hair follicles have taken place.

The histology of this group of soft *nævi* (benign tumours, see Plate XLII) requires careful consideration in order to explain the structure of the malignant melanomata. The histological structure of the various clinical varieties differs very little except in minor points, so that a separate description of each will be unnecessary.

The epidermis varies considerably in different moles. The down-growths of the epidermis usually give rise to the formation of epithelial pearl-like bodies owing to degenerative changes of the superficial layers. Occasionally increased cornification is seen similar to hard moles. The rete Malpighi is thinner than normal and frequently deeply pigmented. The corium is sharply demarcated from the epidermis, and the basement membrane can be seen. In the corium, numerous unpigmented cells are arranged in parallel columns, or in an alveolar manner, lying quite apart from the epidermis and supported on all sides by the fibrous tissue of the corium (collagen). These cells are called *nævus cells*, but the term *angioblasts* is more appropriate as it indicates their origin. The *angioblasts* are oval in shape, and sometimes flattened by pressure. They consist of a clear cytoplasm with a large nucleus, and well marked nucleolus,

occasionally a large cell may be seen with 3-4 nuclei, these angioblasts, especially those which are situated in the neighbourhood of the melanoblasts, not infrequently contain a dirty yellow pigment; I regard this pigment to be ingested by the angioblasts as the result of phagocytosis.

Besides these cells numerous fusiform branching cells are seen containing melanin. These melanoblasts are often so deeply pigmented that the nucleus is not visible, but when it is, it presents the ordinary characters of a resting nucleus. The melanoblasts lie quite apart, and usually along the periphery of the columns of angioblasts. Blood vessels are few and poorly developed. During the growth of the mole, the cells in the corium multiply and exert pressure towards the surface and sides, with the result that the elastin atrophies. The infiltrated area in the corium is inelastic, and is gradually pushed up with every movement of the body by the contractions of the elastic tissue around it. This process Unna described as 'the birth of the mole.'

As the mole grows the epithelium over it hypertrophies, and is stretched, causing a papillary appearance, later on fibrosis may occur at the base and by its contraction constricts the vascular supply and causes œdema.

In the morphological study of the melanoblasts, the close relationship to the blood vessels was emphasised and in the discussion on the origin of moles, I suggested that the angioblasts were the remains of the endothelium of blood vessels in the aborted mesoblastic papilla. The origin of these angioblasts has been a disputed point since 1890. When von Recklinghausen advanced the view of their endothelial origin, Unna maintained that they were epithelial. As far as my observations go, there appears to be no doubt that von Recklinghausen's view is the correct one, but he has unnecessarily restricted it to the endothelium of the lymphatics, whereas I have extended it to the general vascular endothelium. In support of the view the following reasons may now be advanced:—

- (i) From a study of the primitive pigment sheet the appearance of these angioblasts along with melanoblasts is accounted for.
- (ii) The cells are very like endothelial cells, *i.e.*, they have a vesicular nucleus, a clear hyaline protoplasm, an ovoid shape and are quite unlike any epithelial prickle cell. I

PLATE XLIII



N Spots a pigmented patch, probably similar to the pigmented patches of reptilia, etc.



have never seen prickles (intercellular bridges) between them.

- (iii) The arrangement in parallel columns or alveoli suggests a vascular origin (compare endotheliomata). If this were due to 'ensnaring' as Unna believes and the cells moulded into an alveolar shape by pressure of the collagen, we should expect to see a degenerative change, but this does not occur.

Lastly, when the structure of the malignant melanomata is described we shall see that endotheliomata can arise from moles and must have their precursors in endothelial cells. Ribbert, whilst admitting their mesoblastic origin suggested that the naevus cells were young chromatophores, that had not developed pigment. Against this view the following points can be advanced:—

- (i) A study of the comparative histology of the melanoblasts shows that they are specialized connective tissue cells, and not derived from the endothelium.
- (ii) The angioblasts never become fusiform, or branched, and are therefore morphologically distinct from the melanoblasts.
- (iii) Angioblasts may contain pigment if in close proximity to melanoblasts, but the melanin is always a muddy colour as if it was being destroyed by these cells.

#### CHANGES OCCURRING IN SIMPLE MELANOMATA.

The usual sequence of events in the life-history of these neoplasms is that they increase in size until they reach maturity, and then remain quiescent. Growth occurs during the first three decades of life, and again the moles may become active in the sixth decade. Rarely the following changes occur in them:—

- (i) Fatty degeneration—this is only observed in the naevoid cells of melanomata, especially in elderly persons, and probably is synchronous with the general fatty degeneration of the vascular endothelium which occurs in arteriosclerotic subjects.
- (ii) Mollusciform change, previously described.
- (iii) Malignant change. The moles that are apt to become malignant are the large dark ones situated in positions where they are likely to be irritated.

When this malignant change does take place it occurs clinically in one of the following ways:—

- (i) The quiescent mole may suddenly take on growth. From being flat, or verrucose, it becomes papillomatous, dark purple in colour and bleeds readily on the slightest injury: the latter sign is usually the first manifestation of malignancy. The lymphatic glands are soon noticed to be enlarged, and concurrently with this numerous secondary nodules spring up round the primary tumour. In a few months time, general dissemination occurs, and death takes place within 18 months of the first evidence of malignancy.
- (ii) The mole may slowly increase in size, without any bleeding, or ulceration occurring, and the first thing that brings the patient to his medical attendant is the appearance of enlarged glands or numerous secondary deposits in the skin.
- (iii) There may be no change in the primary growth until general dissemination occurs. It has been thought that many of the cases reported, as primary melanotic sarcoma of the ovary, testis, and liver, ought to be included in this group. It is easy to see how a single primary growth which is situated on the back or posterior surfaces of the limbs may be overlooked in a post mortem examination. Such a case is quoted by Fagge; during a post mortem, a primary growth was not at first found to account for the general dissemination, but on more careful examination this growth was discovered in the anal canal. This clinical group is extremely rare, but it is as well to bear it in mind. Structures like the ovary and testis produce varied and complex neoplasms. These organs arise from the genital gland which in turn is derived from a surface downgrowth of the epiblast, growing through the intermediate cell mass to project into the cœlom. It is quite possible for primary melanomata to arise in these situations. In an ovarian teratoma, definite melanoblasts were seen lying in the corium, and if they can occur in these growths there is no reason why primary melanomata may not also occur.

- (iv) On the face, Eve has described a growth which he has named "The Melanotic Rodent Ulcer." It is locally malignant and numerous small black nodules appear round the primary growth: these nodules finally ulcerate and give rise to a rodent ulcer, which is dark in colour. From my interpretation of the histological report it appears to be an ordinary rodent ulcer occurring in or near a mole, which set up proliferation of the melanoblasts. Unfortunately, I have not had the opportunity of examining this clinical group histologically, and therefore can only include it provisionally.

### SECTION III."

#### *Situation and origin of Malignant Melanomata.*

(A) From structures derived from the primitive pigmented pia-arachnoid.

- (a) The uveal tract of the eye. The distribution here is curious as they are ten times more common in the choroid proper than in the ciliary body and are exceedingly rare in the iris.<sup>1</sup> They occur commonly between the ages of 40-60 and are rare in children, in whom gliomas are the common tumours met with.

- (b) The spinal pia-arachnoid. A case was reported by Hirschberg.<sup>2</sup>

- (c) Ogle<sup>3</sup> also reported a case of a Melano-sarcoma arising in the region of the pineal gland. A pigmented tumour arising in this region, at once suggests the possibility of its origin from the third eye, as seen in the *Hatteria punctata*.

(B) From invaginations of the primitive pigmented vascular sheet by surface downgrowths.

- (a) At the Proctodæum. In man these tumours are occasionally seen around the anus and more rarely on the penis.<sup>4</sup> In women, they occur on the special parts of the vulva, viz., Mons veneris, Labia majora and Perineum. Pigmentation of the vagina and cervix is often seen in Indian women, although, as far as I am aware, no cases of melanotic sarcoma have been recorded, as growing from this situation.

- (b) At the Stomatodeum. Sir Frederick Treves<sup>5</sup> describes a case growing from the hard palate.
- (c) At the primary mammary ridge. A few cases have been observed arising in the nipple or accessory nipples.
- (d) Very rarely in connection with the ovary and testes.

Out of thirty-four cases examined, 24 from the choroid, 3 from the anus, 2 from the vulva, 1 from the penis, and four cases from the anal region of horses, all contained spindle-celled melanotic sarcomata.

(C) From remains of this sheet, *viz.*, pigmented patches, moles, etc.

- (a) From moles. Moles have previously been seen to occur as simple pigmented patches (simple melanoma) or aborted mesoblastic papillæ (Melano-endotheliomata). A difference of opinion occurs regarding their structure. Out of 29 growths arising from moles examined histologically, 9 I regarded as spindle-celled sarcomas; one as a mixed growth, partly a peritheloma and partly spindle-celled sarcoma; one from a soft mole as a pure endothelioma; the remaining 18 as a mixed type of growth (Melano-endothelioma maligna).

- (b) From stimulation or implantation of potential melanoblasts of the dermis.

- (i) Xeroderma pigmentosa.<sup>6</sup> In this skin disease there are formed in the first or second years of life, rarely later, numerous small spots which grow paler in winter and darker and larger in summer. In a case recorded by Elsenberg<sup>7</sup> the infant was six months of age and any exposure to the sun was at once followed by an eruption of small erythematous patches on the face, neck and hands; these soon developed into freckles and then followed the ordinary course of the disease. The freckles occur chiefly on the face, and between them in a smaller and more scattered arrangement are tiny, spotted and striated telangiectases. From some of these freckles, large brown or black raised tumours arise, and this event marks the entrance of the disease on a more formidable phase of its history, and may practically be called the patient's death warrant. The histological nature of these tumours appears to be very doubtful,

Gussenbauer<sup>8</sup> has described the malignant phase as a Melano-sarcoma alveolare, and Taylor has described it as a Sarcoma teleangiectoides. We find in the skin of the patients all the transitions from pigmented spots (freckles) to Melano-endotheliomata, and the clinical connection corresponds to the histological findings in this group of neoplasm. Indeed, we find every variety of melanotic neoplasm from pigment spots (melanoblasts and blood vessels) and pigmented warts (Benign melano-endotheliomata) up to malignant Melano-endothelioma (Melanotic sarcoma). Between them all, there is merely a quantitative difference in structure and rapidity of growth.

- (ii) Melanotic whitlow begins in or around the nail bed. The inclusion of melanoblasts in this region can be explained by briefly reviewing the development of the nail bed. The nails appear in the third month of intra-uterine life as fields of thickened epiblast on the ends of the digits, and are slowly shifted back dorsally carrying with them their palmar nerves. At the same time lateral curving of the nail occurs and the persistence of the pigment in this region is shown in many of the simians. Sir John Bland Sutton<sup>9</sup> and Jonathan Hutchinson<sup>10</sup> have called attention to this group of melanotic growths. In 10 cases collected, 7 occurred in females, the average age of the patients being 49 years. As regards the situation, 5 occurred on the inner side of the great toe. This situation is one which is subjected to a good deal of irritation by pressure of boots. Of the remaining five cases, 3 occurred on the index fingers in women; the site suggests causation through needle pricks in sewing. A typical case of these melanotic whitlows was reported by Bowlby in the Transactions of the Pathological Society. A woman, aged 55, had for two years noticed a small black patch like a caustic stain on the inner side of the great toe nail. This grew larger until the whole nail was black, the growth then raised up the nail, which fell off leaving a dark coloured ulcer with a good deal of pigmentation around. The ulcer involved the pulp of the toe and the underlying bone. The toe

was amputated, and some time after the femoral glands were noticed to be enlarged. The woman died two years after the operation.

- (iii) Cases have been recorded by Eve<sup>11</sup> and Morris<sup>12</sup> in which melanomata have arisen in the skin from punctured wounds. Galloway<sup>13</sup> has recorded a case which arose on the heel from the irritation caused by a blister, although there was no history of a previous mole in this situation. Lately I have had sent to me for examination a melanoma, which grew under a corn, and was probably stimulated into activity by pressure. (See Plate XL.)

#### STRUCTURE OF MELANOTIC MALIGNANT GROWTHS.

The melanomata that arose from the situations given under (A) and (B) as well as those arising from the simple pigmented patches (Benign Melanoma) were of the nature of a 'Spindle-celled Sarcoma.' (See Plate XLIV.) The reason may be that the melanoblasts in these situations are vestigial cells and that the angioblasts are normally developed to form blood vessels.

In the embryological study of the melanoblasts, I pointed out how the gradual development of these cells occurs, at first they are fusiform, unpigmented cells, gradually assume their typical shape and pigmentation. The same occurs in the cells of a melanotic sarcoma, the young cells are at first ovoid, or fusiform in shape, with a well-marked, ovoid, vesicular nucleus, and nucleolus. During this phase of development, little or no pigment is seen, and the cells often divide by a hypochromatic mitosis. Later on they become bipolar in shape (light brown pigment granules) and make their appearance. These granules increase in number, and in depth of colour, until the cell is one black mass of pigment. In this stage, the cells may be seen undergoing somatic division. The malignant cells never assume the mature type of a normal melanoblast as seen in reptilia; for the oldest cells, although ink black, still retain their bipolar shape. As regards the situation of melanin in these neoplasms, it is important to bear in mind that they can extrude their pigment, which is taken up by the phagocytic cells around the growth, *viz.* — leucocytes and the plasma cells, as well as by angioblasts in mixed types of growth.

# PLATE XLIV.

Section of the *granulosa* of the *ovary*  
of the *mouse*.



Fig. 1. Section of the *granulosa* of the *ovary* of the *mouse*.  
Fig. 2. Section of the *granulosa* of the *ovary* of the *mouse*.



The dense black colour of the internal secondary growths may be explained by their large blood supply when situated in organs like the liver and also by the fact that light does not affect them. Superficial growths are often lighter in colour, and owing to their defective blood supply, fatty or myxomatous degeneration may occur, whilst the number of well-developed melanoblasts are fewer than those seen in the deep-seated secondary deposits. The nuclei of the tumour cells are also worthy of attention as they sometimes exhibit a curious type of vacuolation. These vacuoles may be single, double, or rosette shaped. They are most frequently observed in the superficial papillomatous growths, and gradually increase in size until all that is left of the nucleus is a ghostlike-structure, the chromatin staining very faintly round the edges. I am inclined to the view that these vacuoles are the result of a mucoid degeneration of the nucleus. The malignant melanotic growths are almost wholly cellular in character with very little stroma. Numerous embryonic blood clefts are seen at the periphery of the growth. Slight cellular infiltration is observed, and I have often noticed that the plasma cells near the tumour contain melanin in a partly broken down state. A brief clinical and microscopical description of two unique cases, which I had the good fortune to see at Middlesex hospital, will not only assist in the description of the Melano-endotheliomata, but will also show how the cells which are seen in neoplasms take their origin.

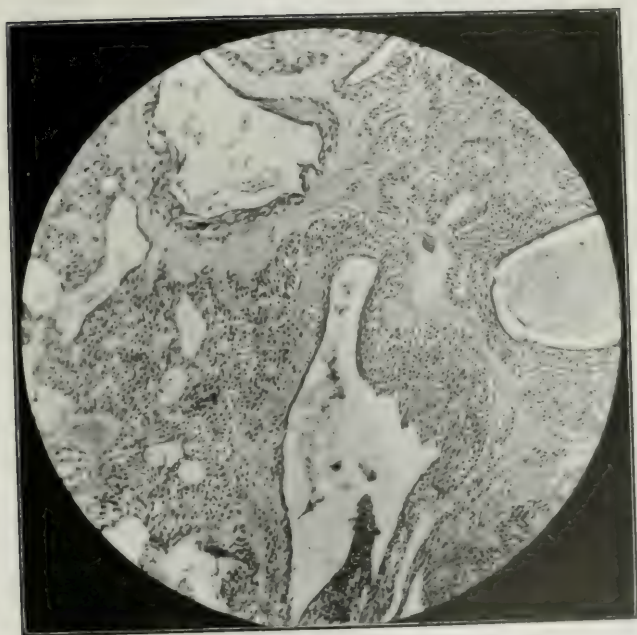
The first case was one which came up to the out-patient department at the Middlesex hospital, and am indebted to Mr. Kellock for being allowed to publish it. The patient was a man of 47. Six months previously he had a soft, white mole excised from his right axilla, as it was rapidly increasing in size and bleeding. When he was examined, numerous slightly pigmented growths were seen in the right axilla, and enlarged glands could also be felt. As he was complaining of pain in the chest and had a cough, he was examined and the signs of a thickened pleura were found. The patient was admitted into hospital and died soon after admission. At the autopsy, the whole of the right pleura, both parietal and visceral surfaces, were covered with unpigmented nodules.

The under surface of the diaphragm and the opposing surface of the liver were similarly involved and numerous white growths were seen on section of the liver.

Microscopical examination of the cutaneous growths (see Plate XLV) showed that the cells forming the growth were arranged in alveoli, and polygonal in shape with a clear reticulated protoplasm. Their size varied, some being large with 3 or 4 nuclei ('plasmodia or giant cells' Sims Woodhead). The nuclei were large and distinctly circular, compare Melano-sarcoma, and contained well-marked nucleoli. Mitotic figures were frequently observed and chiefly of the hypochromatic type. Between the alveoli, the stroma was very thin, consisting of white fibres and in it a few scattered pigment cells were seen. As the extravasations in the growth were numerous, the iron reaction was tried and only the plasma cells in the neighbourhood of these blood effusions were found to contain iron. A few melanoblasts were seen containing pigment and did not contain iron. In the secondary growths from the pleura and liver (see Plate XLVI) the alveoli were smaller and densely packed with the above endothelial cells; the fibrous stroma was more marked and contained elastin; no pigment or melanoblasts were seen in these growths.

This case I regard as an endothelioma, arising from a soft, unpigmented mole, a form of growth which we previously saw contained only naevoid cells. Melanoblasts, which are usually absent from unpigmented moles, were present in this case, but had not shared in the malignant change as shown by the secondary growths. They had proliferated locally as the result of the vascularity and irritation, and were all well developed. The second case was under the care of Sir Alfred Pearce Gould. The patient was an elderly man, who had a large growth, the size of a tangerine orange, growing from the right thigh. It was papillomatous in nature, had a pedicle one inch in circumference, and was almost divided into two parts by a cleft; the larger half was jet black in colour, whilst the smaller was white. Sections were made from each half of the growth. In the light area numerous blood spaces were seen and around them a number of endothelial cells. The angioblasts were many layers deep and cylindrically massed (see Plate XLVII). Between these masses a few scattered, well-developed melanoblasts were to be seen. On approaching the dark area of the section, the blood sinuses became fewer in number and the (cells) endotheliomatous were arranged in layers of 4 to 10 deep around the blood spaces. The melanoblasts predominated in this area, especially towards the periphery of the growth, and were bipolar in shape. Sometimes processes were observed and the cells were entirely obscured by

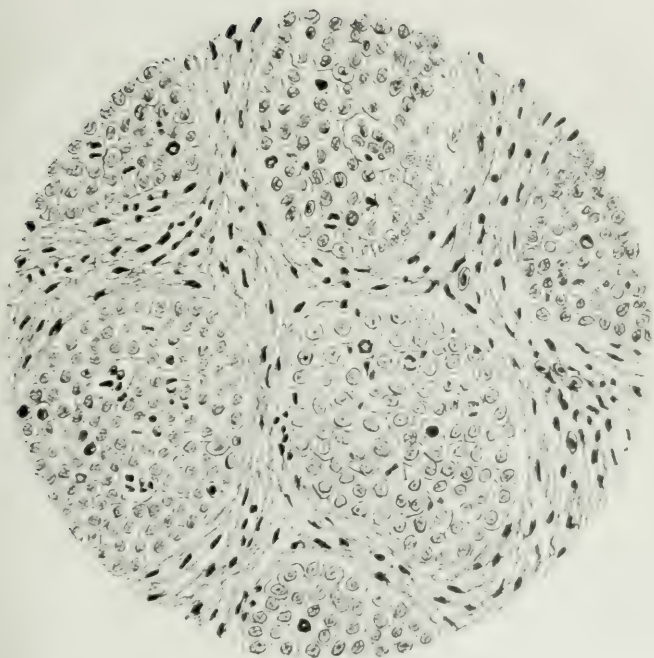
PLATE XLV.



Microscopical section of the cutaneous growth  
from Mr. Kellock's case.



PLATE XLVI.



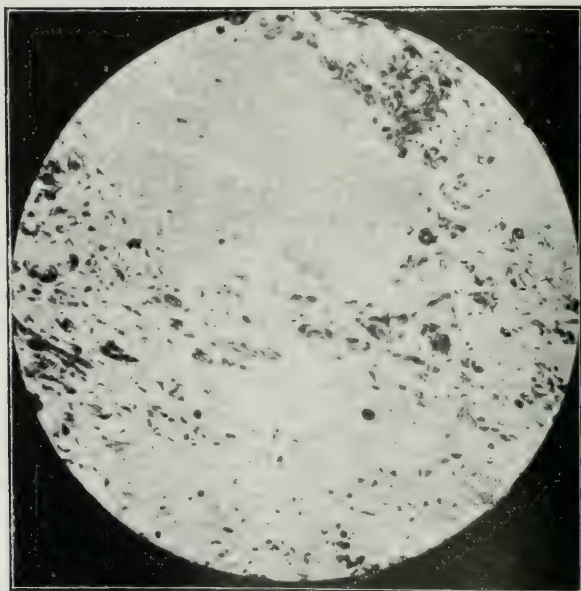
Section of a secondary growth from Mr. Kellock's case  
showing the endotheliomatous nature





PLATE XLVII.

No. 4. 1/2th objective.



Section through the centre of the growth from Mr. Gould's case.

(I) An Endothelial mass.

(II) Numerous mature melanoblasts.

melanin. Numerous young melanoblasts were found towards the centre of the growth.

Here was a growth arising from a mole (Benign Melano-endothelioma) one half of which was peritheliomatous in nature (in white area) and the other half a Melano-sarcoma (in the black area). Now in a mole, there are two kinds of vestigial or aborted cells, lying side by side, and if subjected to the same influence acting on both kinds of cells, may cause a mixed type of growth (Melano-endothelioma). The Melano-sarcomatous element would arise from the melanoblast, and the endotheliomatous from the angioblast or naevoid cell. Therefore, this growth, a rare one, is interesting in the support which it gives to the views expressed in this paper. Mr. Sampson-Handley, in his Hunterian lecture, gave photographs of the light and dark areas of this same tumour, and explained it as being due to a malignant degeneration of the perivascular tissue of a cavernous naevus. (See Plate XLVII.)

In the mixed types of growths (Malignant Melano-endotheliomata) arising from moles it is difficult to identify the cells of the growth, without a previous study of the benign types, *i.e.*, moles. Not only do the two types of cells intermingle with each other so that the typical alveolar arrangement of the endothelioma, and the whorl-like arrangement of the Melanotic-sarcoma is lost, but there is also the difficulty that the angioblasts if situated in the area of diffusion take up pigment granules. If a section of the primary growth of these malignant melano-endotheliomata is studied it will be found to consist of one confused mass of cells, in some parts pigmented, in other parts scarcely pigmented at all. On careful examination, the mass of cells is found to consist of melanoblasts and angioblasts proliferating side by side. In the recognition of these two types of cells the following points were relied upon, *viz.*, the endothelial cells are larger, rounded or polygonal in shape with a circular nucleus; whilst the melanoblasts are smaller, ovoid or fusiform in shape, with a distinct ovoid nucleus and dark staining chromatin. The angioblasts, though normally devoid of pigment, yet, when situated in the neighbourhood of melanoblasts, may contain a few pale yellow granules that have been ingested and broken up into some achromatic substance. The older melanoblastic cells are easily recognized as they are ink black in colour, bipolar in shape and sometimes under a high power are seen to have long processes.

Secondary internal growths present a different structure from the original in so far as they invariably show but one type of component cell, constituting a pure Melanotic sarcoma or a pure endothelioma. This peculiarity of the secondary growths is readily explained by studying the mode of spread. The endothelial cells almost entirely extend by lymphatic permeation, and glandular infection, so in this respect behave more like a carcinoma. The melanoblasts, in addition to lymphatic permeation, quickly invade the blood vessels, especially the veins, and the cells are scattered broadcast by the blood stream. Thus, in mixed types of growths, death occurs long before the endothelial cells have had time to travel far by the slower method of permeation. The life of a patient is only about 18 months with a Melanotic sarcoma, as contrasted with 3-5 years with an endothelioma. Therefore, in the mixed type of growths practically all the internal growths are melano-sarcomatous, owing to the fact that venous embolism plays a large part in the dissemination, whilst the glandular and subcutaneous growths (see Plates XLVIII and XLIX) are often endotheliomatous in nature, owing to spread by permeation. So it does not follow because internal growths are purely sarcomatous, the original growth may not have been of a mixed type. It must further be remembered that a light coloured growth is not necessarily an endothelioma, as it may consist of melanoblasts in an embryonic state or undergoing fatty degeneration.

#### CONCLUSIONS.

(i) Melanoblasts are irregular branching cells, mesoblastic in origin, and are the only cells in the body concerned with the production of the pigment melanin.

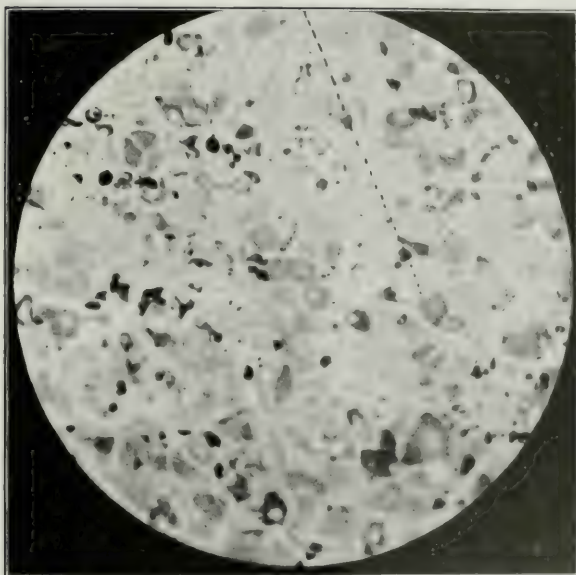
(ii) Melanin is an iron-free pigment formed by the action of an enzyme tyrosinase, which ferment is elaborated by the nucleoli of the melanoblasts.

(iii) In lower animals, the melanoblasts form a large continuous sheet under the skin surface layer, the primitive pigment sheet which is in close relationship to the angioblasts of the cutaneous respiratory capillaries.

(iv) Whenever any surface downgrowth of the epiblast occurs, it carries before it a portion of the primitive pigment vascular sheet thus explaining the pigmented pia-arachnoid of amphibians, and the pigmentation and vascularity of structures formed from the stomatodeum and proctodeum.

PLATE XLVIII.

No. 4. 4th objective

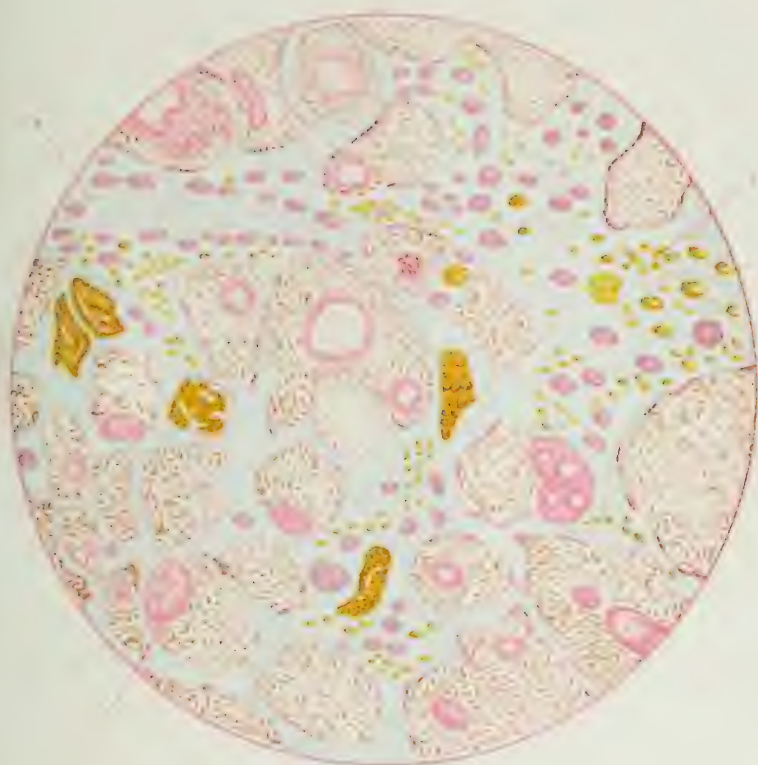


Section of a secondary growth. (Endothelioma arising from a mole from a lymphatic gland.

- (I) Endothelial cells containing pigment.
- (II) Melanoblasts.



# PLATE XLIX



Section of a *Gambusia* embryo, showing the blood cleft  
 (1) Endothelial cells containing phagocytosed pigment  
 (10) Endothelial cells containing phagocytosed pigment  
 (15) Melanin granules free.



(v) There is evidence that the melanoblasts in early life have amoeboid movement, and move from one site to another, *e.g.*, the pigmentation of the peritoneum, etc., of amphibians.

(vi) On the appearance of the dermal appendages, *e.g.*, hooklets, hairs, etc., the melanoblasts become closely applied to them, and their function is largely concerned with the pigmentation of these appendages.

(vii) When hair is formed, the primitive pigment sheet is broken up into longitudinal areas by the primary hair ridges, and then broken up transversely into smaller areas by the hair fields thus accounting for the stripe and spot markings of many of the carnivora. In and between these hair fields, the hair follicles of the lanugo make their appearance, and the melanoblasts apply themselves to these hair papillae.

(viii) In higher mammals, the hairy coat diminishes. In man the hairy coat is almost suppressed, with the result that the melanoblasts are no longer concerned with the pigmentation of hairs, and tend to retrogress, so that in white races they can no longer be identified microscopically.

(ix) In coloured races, the melanoblasts migrate towards the basement membrane, and become thin, elongated and dendritic in order to protect the large skin surface area from actinic rays.

(x) That melanoblasts exist in the dermis of white races can be proved by stimulating them by actinic rays, *e.g.*, bronzing and freckling.

(xi) Albinism is not due to any absence of melanoblasts, but to a congenital anomaly in the secretion of tyrosinase, so that, although the pigment cells are present, no melanin is formed unless the melanoblasts are stimulated into activity, *e.g.*, freckling in albinism.

(xii) A pigmented mole is therefore produced in one of the following ways :—

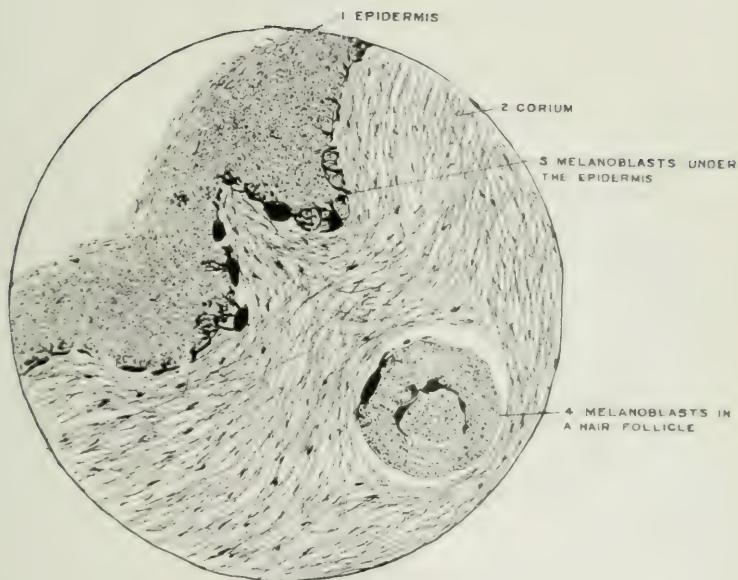
- (1) Benign melanomata consist of an aggregation of melanoblasts under the dermis, and morphologically represent a portion of the primitive pigment sheet of amphibians, etc.
- (2) Benign melano-epitheliomata consist of a papillomatous mass of epidermal cells pigmented by melanoblasts, and morphologically represent a suppressed hair field, or hair area, in which the epiblastic element has largely persisted.

- (3) Benign melano-endothelioma consist of an aggregation of melanoblasts, and angioblasts, morphologically representing the mesoblastic elements of the hair papillæ in which the epiblastic downgrowth has almost wholly or entirely atrophied.
- (4) Benign endothelioma consist of an aggregation of angioblasts, and morphologically represents a suppressed mesoblastic papillæ in which the vascular element alone remains.
- (xiii) The malignant growths are derived in a similar manner.
- (1) Those arising from the melanoblasts only are of the nature of spindle-celled melano-sarcomata.
- (2) Whilst those arising from moles (Benign melano-endothelioma) are necessarily of the nature of malignant melano-endotheliomata and contain both angioblasts and melanoblasts. One or other of these cells may predominate in the primary or secondary growths.
- (xiv) The mode of dissemination confirms the above view. The melanotic sarcoma rapidly erodes the blood vessels, and are spread broadcast through the internal organs, without any glandular enlargement. The endothelioma cells spread by permeation, and less commonly by the blood vessels, and glandular infection and metastatic growths by lymphatic permeation are the rule.

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# PLATE L.



Section of embryo kitten's foot showing the structure of the skin of the pad. (1) Epidermis, (2) Corium.  
 (3) Melanoblasts under the epidermis.  
 (4) Melanoblasts in a hair follicle.



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## SOME REFLECTIONS ON THE KALA-AZAR AND ORIENTAL SORE PROBLEMS.

BY

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[Received for publication, January 27, 1921.]

ABOUT 17 years ago, Sir William Leishman made his important discovery of the parasite of Indian Kala-azar. Since then many workers of all nationalities have contributed valuable papers dealing with the various aspects of these two problems in Tropical Medicine, and to-day the literature is so extensive and written in so many languages that the student beginning research in connection with either of them cannot possibly read them all, and has to depend on current reviews for his information of what other workers are doing. It is true all these papers have been reviewed, but as often as not, the reviewer has perforce had to condense his summary to such an extent that many points which are of importance to the isolated worker are omitted. Further, it is evident that very often the reviewer has not first-hand knowledge of the subject, and is, therefore, not in a position to bring that critical acumen to bear on the entomological, protozoological and experimental sides of the problems; his summary must then fail in that judgment so necessary when the evidence is conflicting. And there are no two problems in Tropical Medicine of a more subtle nature, and calling for wider knowledge of Entomology and Protozoology. Speaking for myself I have always had to read the original papers so as to weigh all the facts and evidence in the light of my own work on the subject. Every worker in India is not in a position to do this, and there is to-day an urgent need for a critical summary of all the work which has been done on the etiology of Kala-azar and Oriental Sore in different parts of the world. As I have been collecting the literature for many years, and have carried out original work

on these two problems, I hope to write such a summary in due course for this Journal. This paper therefore is not meant to be a review of the subject, but just some thoughts and reflections which have been uppermost in my mind during the last few years. As I am not likely to have any more opportunities of investigating either of these two problems in India, these reflections are published with the hope that they may be of some use to other workers on the subject.

The Kala-azar problem has engaged my attention off and on for the last 15 years, and it was very early in my researches that I was confronted with a most serious difficulty in connection with my work on the method of transmission of the parasite to man. I refer to the natural flagellates of insects which belong to the genera *Herpetomonas* and *Crithidia*. It was at once clear to me that a study of the life histories of these flagellates was of the utmost importance, if I was to succeed in following up the extracorporeal life history of the parasite in its insect host. For this reason I have studied some twenty species, and have worked out the life histories of many of them. These studies have been of the utmost value to me in my work on the elucidation of the method by which the parasites of Kala-azar and Oriental Sore gain entrance into man's tissues. As a result of this work I came to the conclusion, in 1906, that the parasites of Kala-azar and Oriental Sore are primary flagellates of insects associated in one way or another with man, and that it is only by approaching these problems from this standpoint that I could ever hope to discover the methods by which they become pathogenic to man.

At the present time it is immaterial to me whether the flagellates of insects are called *Herpetomonas* or *Leptomonas* for I consider the two names are synonymous, and that the question as to which is the correct one depends on the true nature of the flagellate known as *Leptomonas buthli* parasitic in the nematode worm, *Trilobus gracilis*; and this can only be settled by a fresh study of its structure by modern methods. If it is identical in structure with the well-known *Herpetomonas muscae domesticæ*, then all these insect flagellates must be placed in the genus *Leptomonas*. I do not consider that any of the so-called *Leptomomus* of certain authors are distinct from *Herpetomonas*, for the simple reason, that when the life histories of these flagellates in their true insect host are worked out, it is obvious that they exhibit a marked degree of pleomorphism, but that the structure of the mature flagellate always gives the clue to their true nature.

For these reasons I have always maintained that the parasites of Kala-azar and Oriental Sore belong to the genus *Herpetomonas*. It is most unfortunate that Sir William Leishman's name cannot be associated with the parasite of Kala-azar, and no one regrets this more than I do. But the fact remains, as I long ago pointed out, that the parasite of Kala-azar is indistinguishable from many unquestionable *Herpetomonads* of insects. It is true, as Wenyon says, 'The fact that the parasite of Kala-azar has two hosts and can live and multiply in the organs of a vertebrate shows it to be profoundly different from the true *Herpetomonas*, which lives only in an invertebrate. This fact alone would be sufficient to establish the genus *Leishmania*. It is admitted that the *Leishmania* resemble the *Herpetomonas* in their life histories as far as we know them, but in any classification transition forms between the groups occur, as must be so from an evolution standpoint. When a *Herpetomonas* becomes so changed that it has acquired the power of living and developing as an intracellular parasite in the body of a warm blooded animal, and giving rise to such a disease as Kala-azar, we are justified in concluding that it has passed out from the genus *Herpetomonas*, from which it originated, into the genus *Leishmania*.' These remarks formed a strong argument for the genus *Leishmania* when they were published, but to-day they fall to the ground, for Laveran and Franchini have conclusively proved, that more than one species of true *Herpetomonas* parasitic in an insect can live and multiply in the organs of laboratory animals in which they produce lesions exactly similar in every way to those of Kala-azar and Oriental Sore. And further still when smears are made from the organs of animals infected with these parasites, they are seen in the cells as are those of the parasite of Kala-azar in cells in the organs of man, and are indistinguishable. This to my mind is conclusive evidence that the parasites of Kala-azar and Oriental Sore are nothing more than insect flagellates belonging to the genus *Herpetomonas* or *Leptomonas* as the case may be. To continue to look on them as something distinct only tends to obscure the problem. I would suggest that Sir William Leishman's brilliant and far-reaching discovery in human pathology be perpetuated by calling the disease 'Leishman's disease' instead of Kala-azar, which is in most cases meaningless.

#### THE KALA-AZAR PROBLEM.

Knowing then that the parasite of Kala-azar is an insect flagellate belonging to the genus *Herpetomonas*, we are in a position to understand

its probable life history outside man's body. All the known species of *Herpetomonas* are primarily found in the alimentary tracts of their insect hosts, in which they develop and multiply, and eventually reach the hindgut, where they round up, and are then in a state to be passed out in the excreta of the insect. In the case of holometabolous insects this stage is ingested by the larva when it, either purposely, or accidentally, feeds on the excreta of the adult. In its alimentary tract it at once develops into a flagellate and later multiplies. The flagellates are then carried over in the nymph, and later, when the insect hatches out, a certain percentage are already infected, and the flagellates have a further opportunity of multiplying when they are brought in contact with the food of the adult, which in most instances is eminently suitable to them. They now pass down the alimentary tract and eventually round up ready to be passed out in the excreta of the insect.

These species have been in the past veritable traps in any investigation of the extra-corporeal life history of the parasite of Kala-azar. The writer himself once mistook such an insect Herpetomonad in a mosquito for the flagellate stage of the parasite of Kala-azar acquired by the mosquito from the peripheral blood of a case. Fortunately, this mistake was soon recognized by examining mosquitoes which had not been fed on cases of Kala-azar. But many other observers have not been so fortunate and have described true insect flagellates for those of Kala-azar. This has repeatedly happened in the case of the Herpetomonad of fleas, for there is hardly a species of flea which has not its own *Herpetomonas*.

A point of some interest, and which has some bearing on the extra-corporeal life history of the parasite of Kala-azar, is worth mentioning. In the case of those species of Herpetomonads which are parasitic in holometabolous insects which feed on blood, the parasites in passing from the larva to the adult are suddenly brought in contact either with animal or human blood. In the case of these species parasitic in mosquitoes there is clear evidence to show that many of the flagellates are destroyed when they come in contact with the blood in the midgut, and that only those rounding up and round forms survive; these stages are as a rule already in the hindgut of the mosquito, when it hatches out, and the fresh blood hardly, if at all, reaches them, and in any case it does not destroy them.

There are some Herpetomonads which, though parasitic in holometabolous insects, pass their complete life histories in the adult insects alone. A good example of this is to be found in the species of *Herpetomonas*

parasitic in *Stomoxys*. I have never been able to find this parasite in the larva of *Stomoxys*, and there can be very little doubt that the adults acquire the infection when feeding on their hosts, the round stages having been passed out by another fly on the hair. This type of life history is also common to several species of *Crithidia*, and particularly to those parasitic in the Tabanidae and in *Simulium*. Here also the parasites never reach the alimentary tracts of the larvæ of these blood-sucking flies, but are acquired by the adult in the act of feeding. This would undoubtedly account for the extreme rarity of some of these parasites.

There are other species of Herpetomonads which, though normally passing from adult to adult, are equally capable of developing in the larvæ of their insect hosts. *Herpetomonas muscae domesticæ* and *H. mirabilis* are two good examples. The former is parasitic in many coprophagous and necrophagous flies, including species of *Musca*, *Lucilia*, *Chrysomyia*, *Pyrellia* and *Fannia*, to mention a few. It is quite common, however, to find the early stages of this *Herpetomonas* in the alimentary tracts of the larvæ of *Musca*, *Lucilia*, etc. I have elsewhere explained how this happens. The latter is also commonly found in the larva of *Lucilia argyricephala* in which it is capable of growing and multiplying.

Another point which should be remembered in connection with these Herpetomonads of holometabolous insects is that many of them are accustomed to live in symbiotic relationship with faecal bacteria which are common in the alimentary tracts of coprophagous and necrophagous flies, as is the case with *Herpetomonas muscae domesticæ*. I have, however, invariably found that when the bacteria gain the ascendancy, and literally swarm throughout the whole alimentary tract, and more particularly when they are found massed together in the hindgut and rectum of *Musca*, a not uncommon finding, no Herpetomonads can be found in the alimentary tract of the fly. That such bacteria are extremely inimical to these Herpetomonads will be evident when an attempt is made to cultivate *Herpetomonas muscae domesticæ* in pure culture from the alimentary tract of *Musca* or *Lucilia*. If any of the bacteria are inoculated with the flagellates into the NNN medium, the flagellates disappear in two to three days. Whereas, if only the flagellates are placed in the medium, they soon multiply to an enormous extent. It is therefore clear that, although these flagellates can live in the midguts of their hosts with bacteria, the moment the latter gain

the ascendancy the former disappear. A study of those species which are parasitic in blood-sucking flies confirms the view that these particular flagellates cannot live with bacteria in the alimentary tracts of their hosts.

In the case of the *Herpetomonads* which are parasitic in hemimetabolous insects, we find that here again the parasites are ingested in their round stages by the larvæ and nymphs of the insect host. In the case of the gregarious insects, such as the plant bugs, many species of which are infected with *Herpetomonads*, the larvæ and the first nymphs cannot help becoming infected when feeding in company with the adults, and it is in them that the most intense multiplication takes place. In all such insects the food of the larvæ and nymphs is the same as that of the adults. It would be interesting to know the effect of human blood on the *Herpetomonads* of purely plant-feeding bugs.

Bearing in mind these facts regarding the life histories of the flagellates of the genus *Herpetomonas*, we are in a position to consider the probable way in which the parasite of Kala-azar completes its life history and gains entrance to man's tissues.

There are two possible routes by which the parasite can leave the human body:

- (1) Through the alimentary tract.
- (2) Through the agency of a blood-sucking insect.

(1) *Through the alimentary tract.*—This hypothesis assumes that the parasite can pass out of the tissues of an infected person, either in a round stage, or as a flagellate. The only way the parasite can leave the human body would be in the feces. It is well known that many Kala-azar patients at one time or another, in the course of the disease, suffer from inflammatory conditions of the large bowel; and such attacks are often extremely severe and end fatally. During the course of these terminal inflammatory conditions there appears to be a great multiplication of the parasite taking place in the tissues, for I have repeatedly found that at this time there may be immense numbers in the circulating blood. It is assumed that the parasites have invaded the large bowel where they multiply, and there is the possibility they may be passed out in the feces where there should be no difficulty experienced in finding them. I have repeatedly examined the mucus and blood which such patients pass, but have never once been able to find a round parasite. Further, by keeping this blood-stained mucus under suitable conditions, I have never been able to observe any developmental forms of the parasite. Is it passed in some

unknown stage, which has not yet been recognized, and will this stage develop into the typical flagellate if it finds the conditions suitable? One would think that this should happen if such blood-stained mucus is kept in a sterile tube in a cold incubator, but I have failed to find anything of this nature. I am therefore forced to the conclusion that the parasite is not discharged in the fæces of a patient in the round stage.

It would be easy to understand that if it were, it might be ingested in food and drink by a healthy person, and thus find its way into his blood. But this would assume that the parasite can live in the gastric juices. In this connection it should, however, be remembered that laboratory animals have been infected with the Herpetomonads of fleas by allowing them to eat the fleas, although the exact way in which the parasite passes into the blood from the fleas is by no means clear; it is at least possible that they do so through the gastric mucous membrane. Infection may, however, take place in the mouth in the act of eating the fleas. I have repeatedly tried to infect monkeys (*Macacus sinicus*) by feeding them on mucus from cases of Kala-azar, also on heavily infected spleen juice; these experiments which were carried out in 1912-13 in Madras proved negative, and were not at the time recorded. In the case of Indian Kala-azar there seems to be no evidence, either from microscopic findings or from experimental infection, to support the view that the parasite is passed out in the round stage in the fæces and ingested in food by a healthy person.

Can the parasite live in the fæces and be ingested by some sopro-phagous insect associated with man? The fact that no one has found either the round or flagellate stage in fæces, and that the parasite cannot live for any length of time in a test tube in a bacterially contaminated medium is strongly against such a possibility. The only insects I know which might become infected with the parasite are the house flies, *Musca nebulo* and *Musca humilis*. The parasite cannot, however, live in the alimentary tract of the house fly, as I have already shown in an earlier paper.

Taking all these facts into consideration, I am forced to the conclusion that the parasite of Kala-azar if passed out at all in the fæces cannot possibly live in it, and later be ingested by a healthy person. I am well aware of the fact that this method of infection is regarded by some to be one, if not the only, way the infection is acquired in other Kala-azar areas, for instance the Anglo-Egyptian Sudan; I consider the evidence on which this view is based to be misleading. If the parasite of Kala-azar passes

from man to man in this way, we must assume it is no longer an insect flagellate. I fail to understand how any unknown stage is passed in the fæces, and then reaches some aquatic insect. It is difficult to find the parasite in an insect fed on the peripheral blood, and I would imagine it would be well-nigh impossible to find it in an insect fed either on the fæces, or on water containing the fæces.

(2) *Through the agency of a blood-sucking insect.*—We now know that several insect flagellates of the genus *Herpetomonas* are capable of living and multiplying in the tissues of laboratory animals, and there seems to be no reason to doubt that at least one species parasitic in a flea at times finds its way into the tissues of the dog. These parasites are capable of producing a diseased condition exactly similar to the well-known Kala-azar. These facts in my opinion strongly support the view that the parasite of Kala-azar is in the same way a true insect *Herpetomonas*, and that it finds its way into the tissues of man through the agency of its insect host. In the case of the *Herpetomonads* of fleas, which can live and multiply in the organs of animals, there is no difficulty in finding the parasites in their insect hosts under natural conditions. But in the case of the parasite of Kala-azar, on the other hand, it can only be found in a blood-sucking insect if it is fed on a case with parasites in the peripheral blood. In other words the *Herpetomonads* of fleas normally pass their life histories in their insect hosts, passing from flea to flea, and occasionally entering the tissues of animals, but the parasite of Kala-azar is not, as far as we know, transmitted in nature from one insect to another of the same or another species; it is only found in man in nature and in its insect host when fed on man.

Let us consider what insect is the most likely invertebrate host of the parasite. A blood-sucking insect can only become infected, provided the parasite occurs in the circulating blood. I have shown that in Madras, the parasite can always be found in the circulating blood and in certain cases it occurs in large numbers. And it has been shown by culturing the peripheral blood of cases of Kala-azar that the parasites, although in the protoplasm of the white blood cells, are in a suitable condition to develop into flagellates; this change can also take place in the midgut of an insect, provided it is the suitable invertebrate host. The blood-sucking insects associated with man in such a Kala-azar area as George Town, Madras, are as follows :—

(1) Mosquitoes.

(2) Sand Flies (*Phlebotomus*)."

(3) Fleas.

(4) Lice.

(5) Ticks.

(6) Bugs.

(1) *Mosquitoes*.—If any mosquito is the suitable host of the parasite it should be possible to find it developing in its midgut after a feed on the blood of a patient containing many parasites. Although I have carried out such experiments, I have never been able to find the flagellates and I have experimented with more than one species of mosquito common in George Town. Such an investigation requires to be undertaken with the knowledge that mosquitoes are often infected with their own *Herpetomonads*, which they acquire in their larval stages. To exclude such parasites it is not sufficient to collect the larvæ, but to breed them from eggs in water, which is not likely to contain the round stages passed out by the adult mosquitoes.

Perhaps it would be as well to repeat my experiments under favourable conditions in another locality, but Mackie's work in Assam suggests that the mosquito there plays no part in the transmission of the parasite. In any case, the fact that the infection in such an area as George Town, Madras, in which the disease is localized to certain streets, and even to groups of houses, seems to exclude the probability of a flying insect as being the invertebrate host of the parasite.

(2) *Sand Flies (Phlebotomus)*.—Here again in any experiments with these small blood-sucking flies it is necessary to remember that at least two species are known to harbour natural *Herpetomonads*. The evidence in favour of the sand fly being the invertebrate host of the parasite is not very convincing. It is true the sand fly tends to remain in houses near its breeding grounds, and is localised to certain areas, but in George Town the number of sand flies is very limited, the only species being *Phlebotomus minutus*. I have made many attempts to collect these flies from George Town, but have never been able to get them, and the people do not seem to know them; they must therefore be scarce and could hardly account for the many cases of Kala-azar which occur annually.

As I have never fed sand flies on the peripheral blood of cases of Kala-azar, I am not in a position to say whether the parasite develops in the midgut of this insect. Mackie, although he records finding *Herpetomonas phlebotomi* in 10 per cent of specimens examined in Assam, was not able to observe the development of the parasite of Kala-azar in 384 sand flies

fed on the peripheral blood of cases of Kala-azar. He concludes by saying 'The only insect which has given any return for work put into it is the sand fly, and I am of opinion that the relation of this insect to the disease would repay further investigation.' I presume he refers to his finding this insect infected with *Herpetomonas phlebotomi*. This Herpetomonad, as he points out, is clearly a natural parasite of *Phlebotomus minutus*, and I have little doubt is acquired in its larval stage: the larvæ of *Phlebotomus* feed on the feces of other insects and there is no reason to suppose that they would not eat that of the adults, and thus become infected with the round stage of the parasite.

I have examined a large number of *Phlebotomus minutus* caught around the King Institute, Madras, but have never been able to find this *Herpetomonas* in a single specimen. I have seen it stated that Mackie originally found this parasite in Madras, but this of course is not true; he found the parasite in Assam.

It is interesting to note that Laveran and Franchini have recently succeeded in producing a general infection in a young dog by inoculating it with a species of *Herpetomonas* from *Phlebotomus papatasi*.

In my opinion the sand fly in Madras, at any rate, cannot be seriously considered as the invertebrate host of the parasite of Kala-azar.

(3) *Fleas*.—The important and only house flea in George Town, Madras, is *Ctenocephalus felis*, common on the dog and the cat; it is infected with its own species of *Cerithidia*. I had a unique opportunity some years back in dissecting a large number of this species, which had fed on the blood of a dog, which contained many hundreds of parasites of Kala-azar, as many as 500 being seen in each blood film. These dissections clearly proved that the parasite does not develop in the midgut of this flea, strongly opposing the hypothesis that the flea is the invertebrate host of the parasite. I consider it would be a mere waste of time to carry out any further experiments with this flea. As *Pulex irritans* does not occur in Madras, it cannot play any part in the transmission of the parasite; in India this species is only found in the hills. I have, however, fed some *Pulex irritans* on a rich culture of the parasite, but beyond finding the flagellates in their midguts 24 hours after the feed, they had disappeared in all the fleas examined later.

(4) *Lice*.—Early in my investigations on Kala-azar, I fed both body and head lice on cases of Kala-azar, in whose peripheral blood there were many parasites. Although I was able to find the unchanged parasite,

I was never able to find any developmental forms. These experiments convinced me that lice are not the invertebrate hosts of the parasite.

(5) *Ticks*.—I have fed *Ornithodoros savignyi* on the peripheral blood of cases of Kala-azar in Madras, but have never been able to observe any developmental changes of the parasite in its alimentary tract. This is the only tick which is likely to feed on the blood of man, and it never occurs in George Town, Madras. I consider it would be mere waste of time experimenting with any species of tick.

(6) *Bugs*.—Bugs belonging to two genera *Conorhinus* and *Cimex* are known blood-suckers in India, the former as far as I am aware does not suck the blood of man, but probably feeds on the common house squirrel, and therefore has not the same habits as its well known ally, *C. megistus*, in South America. *Conorhinus rubrofasciatus* commonly enters houses apparently attracted by a light, and if carelessly handled will not hesitate to pierce the skin with its powerful proboscis. I bred this bug for many years in Madras in test tubes. A female was caught and fed on the blood of a rabbit, and it soon began to lay its eggs, and in this way large numbers of larvæ were obtained, and these were bred to maturity by feeding them on the blood of a rabbit. A large number of the early stages were fed on the peripheral blood of a case of Kala-azar in films of which there were immense numbers of parasites. On dissecting them at intervals, I found that the parasites did not develop into flagellates, but soon degenerated and disappeared. This, in my opinion, is conclusive evidence that this bug can play no part in the transmission of the parasite in nature; and my observations have been fully confirmed by Cornwall and LaFrenais. Further, *Conorhinus rubrofasciatus* is unknown to the inhabitants of George Town, Madras, and certainly cannot be found there except stray adult specimens attracted to lights.

*Cimex hemiptera*.—This species is the only member of its genus in Madras, and it abounds everywhere. When fed on the peripheral blood of a case of Kala-azar containing many parasites, I found that the parasites slowly developed into flagellates in the midgut as soon as the cells containing them were digested, and that if the bugs were not fed again, the flagellates after multiplying rounded up again. But if, on the other hand, the bugs were given a feed of clean human blood when the parasites were in the flagellate stage in the midgut, they were destroyed and soon disappeared in the vast majority of the bugs. As I was unable to find any stages in the salivary glands of the bug, I came to the conclusion that

the flagellates were reinoculated by regurgitation when the bug fed again. But I now know that this certainly never happens and Cornwall has also proved this. In 1913, I carried out a large number of feeding experiments with heavily infected bugs on monkeys and dogs but the results were entirely negative, and although these experiments were never recorded, I came to the conclusion at that date, that transmission can never take place by the bite of the bug. As a result of my experiences in Mesopotamia, it occurred to me that the allied, though distinct, parasite of Oriental Sore was in that country transmitted to man when the sand fly, *Phlebotomus papatasi*, is crushed on the skin. It seemed possible that the parasite of Kala-azar was transmitted by crushing a bug in the same way, and on my return to India, in 1919, I endeavoured to test this hypothesis. By feeding bugs on pure cultures of the parasite as described by Cornwall and LaFrenais, I was able to observe the behaviour of the parasite, and to confirm my original findings, viz., that when the food in the midgut of the bug becomes exhausted the flagellates appear to round up, and that when bugs heavily infected with flagellates are allowed to feed on clean human blood, in a large percentage the flagellates are destroyed and disappear. If, on the other hand, the flagellates had succeeded in rounding up, they were able to multiply when the bug fed again; this was most marked in the nymphal stages of the bug. Further, it was clear that if a large number of bugs were fed on a rich culture of the parasite containing active flagellates, and the bugs were refed on clean human blood, in the majority it was quite impossible to find any parasites in either the midgut, hindgut, or rectum by examining fresh and stained preparations. Yet in a few they could be found in small numbers after prolonged search. In the majority of bugs which are still infected, they are missed in the microscopic examination in the fresh condition of the contents of the alimentary tracts of the bugs, and in stained smears of the same. Anyone who has examined the midgut, hindgut and rectum of *Cimex* during the process of digestion of human blood will, I think, agree that owing to the length of the alimentary tract and the nature of the contents, particularly of the hindgut, it is practically impossible to find any round parasite in the fresh condition, when in small numbers and as often as not impossible to find one in the stained smear of the contents; in the very process of making and staining the contents the few parasites, which may be present, are lost. The reader may think this is an exaggeration of the case, but I think he will soon change his mind when he searches for such forms, and later

tries to stain them. This is a serious difficulty in connection with the microscopic examination of fresh and stained specimens of the contents of the alimentary tract of *Cimex*.

I realized at once that this method of investigating the problem would never, in the first place, solve the riddle as to how long, and in what percentage of the bugs, parasite persists, and more particularly in what stage, and in what part of the alimentary tract. There could be no doubt that in the majority of bugs, the second, third and even fourth feed of blood, the parasites disappear, but that in a few it persists, and is able to multiply. Were it that the parasite passes to the salivary glands, there would be no difficulty in finding it, but when it remains in the alimentary tract alone it is extremely difficult to do so.

It then occurred to me that the parasite could be most easily detected by culturing the midgut, hindgut and rectum, including their contents in the NNN medium. In order to carry this out, it was necessary to devise a technique which would eliminate all bacteria present on the body of the bug, and without going into details, which have been already described, it was found that by first dipping the bug to be dissected in ether for a few seconds, and then brushing its body thoroughly in 80 per cent carbolic acid, and washing it in five changes of sterile saline solution, its alimentary tract could be safely dissected out without any fear of contaminating the NNN medium with bacteria. Employing this technique I have cultured the alimentary tracts of bugs fed on living flagellates of *Herpetomonas donovani*, and have obtained the following results. It is necessary to note that the portion of the alimentary tract here spoken of as the hindgut is in reality a part of the midgut, that is to say, it is a part of the mesenteron; I have, however, retained the name hindgut throughout.

1. *Bugs fed on cultures of living flagellates showing actively dividing forms, and not refed again on clean human blood; their midguts, hindguts and recta dissected out and placed separately in NNN medium.*

*Adults.*—The midgut and contents of a bug fed in the above way was cultured on the 31st day after the feed on flagellates and gave a positive result. The hindgut, rectum and contents of another bug gave a positive result 34 days after the original feed.

*Larvae.*—The midgut, hindgut, rectum of a bug which had fed as a larva on flagellates gave a positive result on the 12th day after the feed.

2. *Bugs fed on cultures of living flagellates showing at once a dying form, and refed subsequently several times on clean human blood. Their midguts, hindguts and recta were dissected out and separately cultured.*

*Adults.* The midgut and contents of one bug gave a positive result 41 days after the original feed, and after successive feeds of clean human blood. The hindgut and rectum of another bug gave a positive result 34 days after the original feed and with successive intervening feeds of clean blood.

*Nymph.* The midgut, hindgut and rectum of a bug fed as a nymph gave a positive cultural result on the 34th day after the original feed, and with successive feeds of clean human blood.

*Larvae.* The midgut of a bug fed as a larva on flagellates and subsequently fed on clean human blood gave a positive result 12 days after the original feed and with intervening feeds of clean blood.

From these results it will be seen that if adult bugs are fed on living flagellates of *H. domocani*, and not refed again on clean human blood, the parasite can be recovered from their alimentary tracts as long as 31 days in the midgut and 34 days in the hindgut and rectum. The fact that the parasite is able to multiply and show a profuse culture of flagellates in the NNN medium conclusively proves that it was in a living condition in the alimentary tract of the bug at the time the culture was made. The parasite can therefore live for at least 31 days in the midgut of the bug and for 34 days in the hindgut and rectum without any food, for these bugs were not fed again and their midguts were empty, and their hindguts only contained digested blood. I believe this does not represent the limit of time the parasite can survive in a living condition in the alimentary tract of the bug, for as only a comparatively small number of bugs were used in the experiment, and as they were gradually dying, the last one was not kept longer than the 34th day.

The second series of experiments prove that if adult bugs are fed on flagellates of *Herpetomonas domocani*, and if the bugs are subsequently fed on clean human blood, the parasite can survive and has been found in a living condition in the midgut of the bug 41 days after the original feed and in the hindgut and rectum 34 days. It was clear from this particular series of experiments, that in the greater proportion of the bugs the parasites had disappeared having in all probability been destroyed by the fresh blood. The parasite can be found 34 days later in the midgut, hindgut and rectum of a bug which had fed as a nymph on flagellates; and 11 days in a bug which had been fed as a larva.

Here then we have a very clear indication that the parasite of Kala-azar can survive in the midgut of *Cimex hemiptera* for a long period in spite of refeds of human blood. There can be no possible fallacy in these cultural experiments. In each case the midgut, hindgut and rectum was dissected out and separated from the remaining part of the alimentary tract and transferred to a tube of NNN medium.

One of the most puzzling facts connected with the spread of endemic Kala-azar is that the infection persists in a house, or a locality for long periods, and it has been difficult to explain this phenomenon from existing knowledge of the extracorporeal life history of the parasite. But now that we know that the parasite can persist in an insect in spite of repeated feeds of human blood, which at one time I believed destroyed it, we have a clear solution of this puzzling phenomenon. Further, as it is only a small percentage of bugs, which, on becoming infected, can remain so, we are in a position to explain why the disease is not more prevalent although the bug abounds everywhere. That is to say, we can explain why the distribution of the disease does not correspond with the distribution of the bug.

I have little doubt that when these experiments come to be repeated by other observers, the time that the parasite can persist in the bug will be found to be longer. I consider these results most encouraging as they have opened up a new method of attacking this problem; they have been obtained by feeding bugs on the flagellate stage of the parasite, and it now remains to feed bugs on the peripheral blood of a case of Kala-azar containing many parasites. I would suggest the following line of enquiry as being the most likely to lead to successful results.

A large number of adult bugs (*C. hemiptera*) should be collected and fed on healthy human blood, all their eggs collected later and the larvæ kept in a starved condition ready to be fed on the peripheral blood of a case containing many parasites. Some may be fed once only on the case and others several times. Each lot should then be fed on healthy human blood, and their midguts and rectums dissected out and cultured as described above, and the culture tubes kept at 22°C; some may be fed on clean blood until they reach the adult stage and then their alimentary tracts cultured. By carrying out such a series of experiments we would then know the extreme limit of time the parasite could live in the various

parts of the alimentary tract, and more particularly in which part longer than another.

But such an enquiry must be carried out with the greatest care, and it will be very evident, that it demands a high degree of manipulative skill in dissecting out the alimentary tract of the bug without rupturing it, and without contaminating it with bacteria; these must be excluded from the medium if we expect to obtain cultures of the parasite. Each step in the enquiry must be considered as important as another so as to prevent any mistake being made which would cause some of the observations to be negative. The bugs must be bred by an Assistant whose duties are to see that all the stages are ready when required, and that no mistakes be made by allowing fed bugs to die owing to the omission of some small detail. I have found that equally good results can be got by using a pipette to suck up the contents, say, of the midgut, especially when it has ruptured, and transferring this to the NNN medium. It may be of some importance to place the contents in one NNN tube, and the gut wall in another.

If such an enquiry were carried out in a Kala-azar area for about two years using only peripheral blood feeds, and only those cases showing many parasites in blood films, I am convinced some valuable clues as to the next step in the enquiry would be discovered. We would, for instance, find out the percentage of bugs in which the parasite persists, after long periods with feeds of clean human blood following the infected feed. We would also know which stage of the bug is most suitable for the persistence of the infection. Having found out these facts, we would be in a position to examine the bugs microscopically with far greater chances of success than by examining them without this knowledge.

There are one or two points in connection with the life history of *Cimex hemiptera* which call for note here. It will be remembered that this bug has seven stages in its life history, an egg stage, larval stage, four nymphal stages and an adult stage. The following observations on the life histories of *C. hemiptera* and *C. lectularius* were carried out at the Pasteur Institute, Coonoor, by Laboratory Assistant Uja under my supervision. The bugs were fed on himself, and accurate observations were made on each bug noting whether it had fed or not. The results may be taken to represent what would take place in Nature, under similar conditions of temperature, etc.

Life history of *Cimex hemiptera* Fabr.

Kept at average maximum temperature 68°F.

and average minimum temperature 65.7°F.

Humidity at 8 hours—84; the bugs applied every day for feeding.

		LARVAL AND NYMPHAL STAGES.		
		Number of feeds.	Number of days in changing from one stage to another.	Number of days from larval to last nymphal stage.
Larval stage	..	3	9 to 12	66 days.
1st Nymph	..	3	11 to 14	
2nd Nymph	..	4	13 to 15	
3rd Nymph	..	4	14 to 16	
4th Nymph	..	5	16 to 19	

Adult stage.

Number of days from last nymphal change to egg laying.	Number of feeds.	Eggs. Number of days to hatch.
22 days.	5	15 to 17

Life history of *Cimex hemiptera*; kept at 91.2°F.

Applied daily.	Number of feeds.	Number of days to change from one stage to another.	Number of days from larval to the last nymphal stage.
Larval stage	1	3	21 days.
1st Nymph	1	3 to 4	
2nd Nymph	2	3 to 5	
3rd Nymph	2	4 to 6	
4th Nymph	3	5 to 7	

## Adults at same temperature.

Number of days from last nymphal change to egg laying.	Number of feeds.	Eggs. Number of days to hatch.
6 days	3	6 to 7

Life history of *Cimex lectularius* L.

Kept at average maximum temperature 68.0°F.

and average minimum temperature 65.7°F.

Humidity at 8 hours—84.

Applied every day for feeding.	Number of feed.	Number of days in changing from one stage to another.	Number of days from larval to last nymphal stage.
Larval stage ..	3	9 to 11	67 days.
1st Nymph ..	4	11 to 13	
2nd Nymph ..	4	13 to 16	
3rd Nymph ..	4	15 to 17	
4th Nymph ..	5	16 to 19	

## Adults kept at same temperature.

Number of days from last nymphal change to egg laying.	Number of feeds.	Eggs. Number of days to hatch.
23 days.	5	15 to 17

*C. lectularius*, kept at 91.2°F.

Applied every day for feeding.	Number of feeds.	Number of days to change from one stage to another.	Number of days from larval to the last nymphal stage.
Larval stage ..	1	3	24 days.
1st Nymph ..	1	4 to 5	
2nd Nymph ..	2	5 to 6	
3rd Nymph ..	2	6 to 7	
4th Nymph ..	3	6 to 9	

Adults at same temperature.

Number of days from last nymphal change to egg laying.	Number of feeds.	Eggs. Number of days to hatch.
7 days	3	6 to 7

From these observations it will be noted that the life histories of the two species are the same under identical conditions. The larval and nymphal stages feed from three to five times between each stage, and 19 to 20 feeds are taken during the immature stages at the temperature under which the experiments were carried out. As far as I am aware *Cimex* in all its stages only stops feeding when food is not available, and when it is infected with bacteria, the latter a very rare occurrence, if ever, in Nature. It is true under natural conditions bugs will be found in all stages in a bed, and it is evident some do not feed as often as others, most probably owing to lack of opportunities. A bug feeding on a case of Kala-azar would continue to do so until it was removed or killed. If the parasite is to develop and remain in the alimentary tract, it must, therefore, be able to resist the fresh feeds of blood.

Let us now consider how the bug can infect man. If the parasite does not pass to the salivary glands, and at present there is no evidence to show that it does, the only other way it can be transmitted by the bite is by passing up the oesophagus into the pumping organ, and is then regurgitated when the bug sucks blood. This, I now know, never takes place, and further I have never succeeded in finding the parasite either in the pumping organ or the proboscis. The only other way it can pass to the skin of man is when an infected bug is crushed on the skin. Bugs are very frequently crushed, and I have been repeatedly told so by Kala-azar patients. Recently I had two little girls suffering from the disease under my care. They had contracted it at the Railway quarters in Madras, where they were living with their parents in a block of rooms. In one of the rooms, close by, a man died of Kala-azar, and a month or so before this event took place, he had been bedridden suffering from terminal dysentery, and the mother of the girls told me that they very frequently went to his bedside and talked with him. His bed was heavily infested with bugs, as were also those of the two little children; in fact the mother

told me that she found it impossible to get rid of them, there were so many. She further said it was quite common to find marks of blood on the necks, ears, hands and legs of her daughters, and that there was no doubt these represented the blood from crushed bugs. In order to reach the children they had to crawl out of the beds over the bed clothes and then were able to reach the skin. It was evident they were crushed by the children during sleep, the irritation directing the hands to the spot when the bug was crushed. All this information was given by the mother without my putting any leading questions to her.

The larval and nymphal stages of the bug are those which are most readily crushed. It is only necessary to let some feed to repletion, and then to apply slight pressure to their bodies when it will be found that they at once rupture. It is almost impossible to dissect out the midgut of a larva, or a first and second stage nymph without rupturing it, when it is fully fed with blood.

Let us now consider in what stage the parasite is likely to enter the human skin. But before discussing the various possibilities, I will refer to a recently discovered stage in the development of the parasite in the bug. It will be remembered that Cornwall recently described a peculiar phase in the flagellate stage of the parasite, which he has named the 'thick tail.' He has pointed out that this stage is only seen in the alimentary tract of the bug, but is also found by mixing a drop of culture containing flagellates with the alimentary tract of the bug. He makes the following remarks regarding the conditions which determine the appearance of this form. 'It has never been seen in ordinary cultures of the flagellate, nor when cultures are mixed with serums, saline solutions, acids, or alkalis. If, however, the stomach of a *Cimex* be dissected out and teased up on a slide and some flagellates added from a culture, and the preparation be sealed with vaselene, it will be found on examination next day that a certain number, perhaps a hundred or more, of the flagellates have become thick tails. The transformation occurs only in actual contact with a piece of stomach mucous membrane; thick tails are never found in the outlying fluid of the preparation where the majority of the active flagellates congregate. There does not seem to be any particular attraction in the mucous membrane for the flagellates; indeed they often appear to be fewer in the immediate neighbourhood of fragments than at a distance. Not every preparation of bug stomach and flagellate culture is successful in producing thick tails; just as one fails to find thick tails in every bug which has been fed on flagellates.

It does not appear that the condition of the stomach makes much difference, whether it be taken from a starving bug or one recently fed. It does seem, however, that the presence of nutrient matter favours the formation of thick tails, for more successful preparations were obtained when a little fresh human blood was added to a starving stomach than when nothing was present but stomach salt solution, and a drop of flagellate culture; as also when the blood, already present in a distended stomach, was retained in the preparation instead of being washed away.' He goes on to say that, 'Thick tails are nearly formed as well when the intestine of *Cimex* is used instead of the stomach, but not when muscle and bits of other tissues are present. Other experiments with both *L. donovani* and *L. tropica* were made with the stomach of rabbits, rats, fleas (*Xenopsylla*), *Conorhinus rubrofasciatus*, and ticks (*Margaropus*), all with negative results. The mucous membrane of the stomach and intestine of *Cimex rotundatus* alone caused the transformation.'

It will be noted from these observations of Cornwall that the reaction which results in the formation of the 'thick tail' is of a specific nature, contact with the cells of the alimentary tract of *Cimex* alone producing it. As soon as I saw this stage of the parasite in the bug, I recognized it was some thing I had not seen previously in my feeding experiments in Madras. There could be very little doubt that it played some important part in the life history of the parasite in the bug, and I concluded that it represented some particular stage in the multiplication of the parasite in the bug. I made no further observations on the genesis of this stage.

What then is the significance of this stage which, if once seen, cannot be mistaken again? Before answering this question, it is necessary to record an important discovery made by Mrs. Adie in Shillong. At my request, and through the generosity of the Governing Body of the Indian Research Fund Association, under whom Mrs. Adie has been working for the last two years, she came to Coonoor to discuss and show me the results of her work on the development of the parasite in *Cimex*. Mrs. Adie has succeeded, by feeding bugs, *Cimex lectularius*, on splenic juice, from cases of Kala-azar, in discovering an intracellular stage of the parasite in the above bug. This stage is the thick tail first discovered by Cornwall in 1916. She has observed flagellates in the fresh condition endeavouring to penetrate cells of the midgut by their posterior ends, and has actually observed one enter a cell. She has also observed the

flagellates in cells, and has found, that when once established there, they become the thick tail described by Cornwall. A sheath is thrown out all around the flagellate, which now rounds up, the flagellum being withdrawn into the sheath. Division now takes place and the flagellates may be seen squirming round and round within the sheath: the daughter flagellates leave the cell and swim into the contents of the midgut. Mrs. Adie has pointed out to me that this stage of *Herpetomonas donovani* is very similar to the intracellular stage of *Trypanosoma lewisi* in the midgut of the rat flea, *C. fasciatus*, described by Minchin and Thomson, the flagellate passing into a cell, posterior end first, in order to multiply.

Mrs. Adie's discovery at once clears up the true nature of the thick tail first found by Cornwall readily explaining the points noted by him, and which I have quoted above. I have been able to confirm Mrs. Adie's discovery and have observed flagellates in cells of the midgut of *Cimex hemiptera*, the process of thick tail formation in the cell, and the division of the parasite within the sheath. As I had no time to feed bugs on flagellates, I devised a simple technique, which, though not good enough to show the parasites in the cells in sections, is an excellent way to see the changes in the fresh and in stained smears of the midgut. After having seen flagellates actively moving in the contents of the upper part of a goblet cell of the midgut, and others burrowing down into the deeper parts of the cell, there could be no doubt whatever of the existence of this intracellular stage. I realise that in order to obtain good sections showing the parasites in the cells, special and delicate technique will be required which will take a long time to carry out; this will be postponed to another occasion. Further, I do not propose describing the changes undergone during the intracellular stage, but will leave Mrs. Adie to do this in due course. I would, however, like to point out here, that as Cornwall noted, the thick tail formation is not seen in the gut of any other blood-sucking insect except *Cimex hemiptera*. This change does not take place with the midgut of either *Pulex irritans* or *Pediculus humanus*. This is then the final proof that *Cimex* is the true invertebrate host of *Herpetomonas donovani*.

It will be clear now that the thick tail is part of an intracellular stage of *Herpetomonas donovani* in *Cimex*. I am not in a position to give the true explanation of why some flagellates pass into the cells in order to undergo a process of multiplication, but it may be that this stage is necessary in order to escape destruction during digestion, when the

bug takes the next meal of blood. It will be remembered that by feeding bugs on the peripheral blood of a patient, I have observed the flagellate stage on the third day, and that if such bugs are fed again on human blood the flagellates disappear from the midguts. But if in some of these bugs the flagellates are already in cells, they are protected, and can multiply, and again reach the contents of the midgut, when the blood is no longer inimical to them. Further, it is quite clear that this intracellular stage does not take place, in every bug, for it is only in the midguts of a small proportion that one finds them. This is what would be expected for it is exactly what happens in the case of *Trypanosoma lewisi* in the flea, *C. fasciatus*. And it will be remembered that in the case of *T. lewisi* the intracellular stage is followed in due course by smaller forms of the flagellates, and it is only when this phase is formed in the rectum of the flea that *T. lewisi* is infective to its vertebrate host, the rat. It is important to note that here the infection is not acquired by the bite of the flea for the flagellates never pass to its salivary glands, the rat only becomes infected when it either licks off the fæces of a flea containing the infective phase, or eats a flea containing these forms. So it is very probable that in the case of *Herpetomonas donovani*, the infective form only follows on the intracellular multiplication, and that this form appears later in the hind intestine and rectum of the bug. I have never found any parasites in the salivary glands of heavily infected bugs, and very few parasites in the rectum, so that I believe the hind intestine in this case is the part selected for the infective stage. How does this reach the skin of man?

I have already pointed out that I believe *Herpetomonas donovani* is acquired by man when an infected bug is crushed on the skin, the infective stage gaining entrance into the puncture produced by the proboscis of the crushed bug, or into a minute abrasion. That bugs, particularly in their early stages, are crushed on the human skin is beyond dispute, so that there seems no difficulty in understanding this method of infection; and I believe the infective stage is a round one.

We are now in a position to explain some of the puzzling phenomena connected with the spread of *Herpetomonas donovani* in India. I have first shown that the parasite only flagellates in the midgut of *Cimex* and not in that of any other insect. Next I have been able to prove by cultural experiments, that the parasite is able to live for a long time in the alimentary tract of the bug, 41 days in the midgut, and 34 in the hindgut, and that these results were obtained in two bugs out of a large

number fed on flagellates ; in the vast majority the parasites had evidently disappeared, so that they could not even be detected by culture. These results clearly indicate that it is only in a small percentage of infected bugs that the parasite is able to maintain itself. Further experiments along these lines might quite well give longer periods of survival in the bug. At any rate we can understand that a larva may retain the infection until it becomes an adult, a period of about 66 days. And we know that the adult *Cimex* can live for many months as such, so that it is even possible that a bug infected as a larva may retain the infection long after the adult stage is reached and this may be many months. The existence of an intracellular stage in a small percentage of bugs would explain how this infective stage is produced. I have also shown that the development of the parasite in the bug is dependent on certain temperature conditions. Taking all these established facts into account we have a ready explanation of why Kala-azar is so localized, and why, although the bug is distributed throughout India, the disease is only found in certain relatively small areas. We also know that it is not every case of Kala-azar which can infect the bug, and that it is only in those cases in which there are large numbers of parasites in the circulating blood that they reach the midgut of the bug in sufficient numbers and there become flagellates.

It has always been difficult to explain why the disease has not spread more than it has outside George Town, Madras, but when we remember, that on the analogy of *T. lewisi* in the flea and *T. gambiense* in *G. palpalis* it is perhaps only about 5 per cent of bugs or even less which become infective. The chances of these five being carried to other parts is small. And we must remember that if the infection is acquired when a bug is crushed on the skin, one bug can only infect one person, whereas one infective *G. palpalis* may infect every person it feeds on. That Kala-azar exists in other parts of the Madras Presidency is certain, and the reason why its exact distribution is not known is obviously due to the fact that even now the cases are not accurately diagnosed. I have been told on good authority that the disease has become established at Rameswaram, and if this is true, its existence there calls for enquiry for it is conceivable it may spread into Ceylon.

Once established in any locality the disease only spreads slowly for the reasons given above which govern its spread. Further work on the life history of the parasite, and particularly the number of bugs which

become infective after feeding on suitable cases, will alone clear up the remaining mysteries connected with its spread ; the lines along which such enquiries should be conducted are, however, now clear, and there should be no difficulty in solving all these doubtful points.

In addition to these lines of investigation, and particularly to the one which I consider is most likely to lead to the discovery of the percentage of bugs which remain infected after being fed on the peripheral blood, it will be very necessary to study the development of the parasite in bugs fed in the natural way on the peripheral blood of suitable cases of Kala-azar in order to study the intracellular stage under these conditions, and the resulting forms which render the bug infective. Mrs. Adie's discovery was made by feeding bugs on splenic juice which contained a large number of parasites, and, like Cornwall, I have recently fed bugs only on flagellates. Although I never saw the thick tail in bugs fed on the peripheral blood in Madras, I have little doubt it will be found in bugs fed in this way. I undoubtedly missed it because I did not examine a sufficient number of midguts in the fresh condition. In the stained smears the thick tail is easily missed unless one knows exactly what to look for.

In carrying out feeding experiments with bugs on the peripheral blood of cases of Kala-azar, it is very important to feed them only on those cases in which there are a large number of parasites in the circulating blood. Several blood films should be taken at the time the bugs are fed, and the number of parasites recorded. From very considerable experience of such experiments, I know that it is quite useless feeding bugs on patients in whose circulating blood there are very few parasites, such as 6 to 12 in each blood film ; only those cases which show from 20 to 100 or more parasites in a film of finger blood should be used for such feeding experiments. This has not been done by any observer in India, but on the contrary unnecessary discredit has been thrown on my work on the development of the parasite in *Cimex* by the recording of dissections of bugs fed on the blood of cases of Kala-azar containing very few or no parasites in films of finger blood. For instance, Mackie states he examined the alimentary tracts of 1,512 wild bugs collected from Kala-azar houses in Assam and failed to find any parasites in them. He also records having dissected 322 young laboratory bugs fed on cases of Kala-azar with negative results, except in the case of two bugs, but no mention is made as to whether films of finger blood taken at the time the bugs were fed contained any parasites. There is no proof that the bugs collected from

the Kala-azar houses had even fed on the diseased people, and much less that the patients' blood contained parasites. It is only to be expected that dissection of bugs fed in this way would be negative. In my opinion such experiments are valueless, and cannot be brought as evidence against the development of the parasite in the bug. In any case they represent very crude methods of investigation. Yet it is these very negative results of Mackie's which are quoted as proof that the bug cannot be the carrier of the parasite in Nature.

Having once worked out the intracellular stage in bugs fed under natural conditions, and determined the part of the alimentary tract where the infective forms finally come to rest, it will be very necessary to try and infect animals. I would suggest that, in the first instance, the part of the alimentary tract containing these forms should be dissected out intact, teased up in sterile normal saline solution, drawn up into a sterile glass pipette, and the gut and contents inoculated under the skin of young monkeys and dogs. Later, infected bugs should be crushed on the skins of other monkeys over a spot where bugs have inserted their proboscides. It would be necessary to examine the bone marrow of all the monkeys both microscopically and culturally at regular intervals, for the disease is often latent in these animals, and no symptoms may develop. Infection of these animals even with rich splenic fluid often fails, so that a large number would have to be experimented with.

I have crushed a large number of infected bugs on the skins of monkeys at the Pasteur Institute, Coonoor, but so far with negative results. But I now see that as the bugs were crushed soon after the infected feed the parasites would in all probability still be in their intracellular stage; and it is most unlikely that this stage would infect any animal, and man for the matter of that.

Before passing on to the Oriental Sore problem, it is necessary to refer to Franca's recent work on the life history of *Herpetomonas doudi* in *Stenocephalus agilis*. It will be remembered that this parasite causes a diseased condition in several species of *Euphorbia*, the parasite living in the latex juice of the plant. Franca finds that the parasite after undergoing a process of multiplication in the alimentary tract of the little plant bug, which feeds on the latex juice of *Euphorbia*, passes to the salivary glands, and that it is introduced into a healthy plant when an infected bug pierces the outer layers of the plant stem. He lays great stress on the local lesion which develops at the site of the puncture made by the proboscis of the bug, and finds the parasites are first

localized before invading the latex juice. I have all along held the view that there is a local lesion in the case of infection with *Herpetomonas donovani* in the skin at the site of the entry of the parasite. This lesion, however, never develops into the characteristic papule produced by *Herpetomonas tropica*, but soon disappears. Such a lesion would be small and easily overlooked.

It is interesting to note that Franca believes that *Stenocephalus agilis* is the true host of *Herpetomonas davidi*. It is very possible also that this species has an intracellular stage in the cells of the midgut of *Stenocephalus*.

#### THE ORIENTAL SORE PROBLEM.

THE fact that the parasite of Oriental Sore is indistinguishable from that of Kala-azar has led many to believe that they are identical, and it has been suggested by Manson and others that Oriental Sore may be but a modified form of Kala-azar analogous to the well known Vaccinia and small pox. There can, however, be little doubt that the two parasites are distinct, their structural similarity only being superficial. The writer knows a case of an officer who contracted Oriental Sore on the Indian Frontier, and later Kala-azar in Assam, so that there does not seem to be any protection afforded from an attack of the former against the latter.

The parasite which causes Oriental Sore in India should be known as *Herpetomonas farunculosa*, as it was first described by Firth in 1891, and like its ally *H. donovani* it is an insect flagellate. In India this parasite occurs on the Western side, all along the frontier and the course of the Indus and its tributaries; its exact eastern limit is not known, but it reaches as far south as Cambay. It is very easily acquired in such a place as Cambay where it is only necessary to spend a night during the cold weather, and six months later a small papule appears on some exposed surface of the body. When in Cambay I was able to investigate a number of cases which clearly proved this. The exact nature of the lesion is never recognized until it has persisted for some time, then medical advice is sought and the real nature of the nodule diagnosed by the finding of the parasite.

I examined a large number of sores and scars in Cambay, and found that they occurred on all parts of the body except the genital organs, the palms of the hand and the soles of the feet. The photographs accompanying this paper, which were very kindly taken by Mr. Avari of the



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

Some typical cases of Cutaneous Herpetomoniasis

W. S. PATTON.—Some reflections on the kala-azar and oriental sore problems.



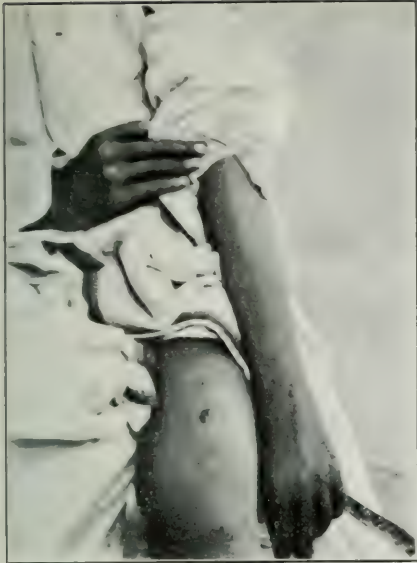


FIG. 1.



FIG. 2.



FIG. 3.

Some typical scars of Cutaneous Herpetomoniasis.

W. S. PATTON.—Some reflections on the kala azar and oriental sore problems.





FIG. 1



FIG. 2.



FIG. 3.



FIG. 4.

Some typical scars of Cutaneous Herpetomycosis.

W. S. PATTON.—Some reflections on the kila azvi and oriental scro problems.





FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

Some typical sears of Cutaneous Herpes zoster.

W. S. PATTON.—Some reflections on the kala azar and oriental sore problem.





FIG. 1.



FIG. 2.



FIG. 3.

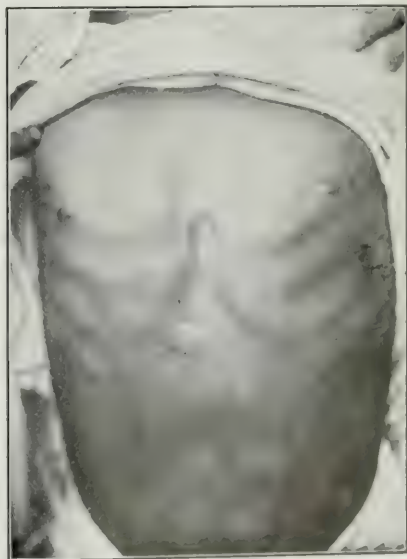


FIG. 4.

Some typical scars of Cutaneous Herpetomoniasis.



Bacteriological Laboratory, Parel, give a good idea of the distribution of the lesion on the body. Plate LI illustrates a number of typical sores. Fig. 1 shows a sore of about six months' duration on the cheek of a girl close to the mouth; it contained large numbers of parasites, and the central part was covered with a thick crust which appears dark in the photo. Fig. 2 shows a large fungating sore on the nose of a little boy; it had persisted for about  $4\frac{1}{2}$  months, and parasites were only found in small numbers at the margins, the whole of the central part having ulcerated. Fig. 3 shows a typical excavated sore on the cheek of a boy near the angle of the jaw; the parasites were recovered in small numbers from the raised edges. Fig. 4 illustrates a very interesting sore on the scalp of a boy, a very unusual site. It had a small central ulcerated area and many parasites were found on puncturing it. The photographs on Plate LII illustrate some typical scars on the legs and arms of school boys in Cambay. Fig. 1 is that of a little boy who had one sore on the anterior surface of the left thigh, and another on the upper surface of the left forearm; it was from the former that the author inoculated himself in 1910. Fig. 2 shows some scars on the arms of two boys; that on the left has one scar on the anterior surface of the upper arm, that on the right a large scar on the dorsal surface of the wrist and another a little higher up. Fig. 3 is a photograph of the thighs of two boys with some typical scars, that on the right has one which has only just healed. Plate LIII illustrates some scars on the face. Fig. 1 is a photograph of a little European boy who lived in Cambay and contracted a sore on his right cheek, and which left an ugly scar. Fig. 2 is that of a youth who had two scars on his face, one on the bridge of the nose and the other on the cheek. Fig. 3 is a photograph of a man who had a scar on the side of the nose, the sore having been contracted when he was a boy, and which, as will be seen, has destroyed a part of the nose. Fig. 4 is a photograph of a fakir who came to Cambay to collect some of the famous Cambay stones to make a chain; he left the town with the seal of Cambay as the scar is locally known, on his left hand. Plate LIV, Fig. 1, shows a typical scar in the centre of the forehead. Fig. 2 another on the ear, also an unusual site for a sore. Fig. 3 shows a large irregular scar on the right temple of a man, and in Fig. 4 are shown some scars on the faces of two boys. Fig. 1, Plate LV, illustrates a large scar on the chest of a boy. Fig. 2 a scar on a boy's back, an uncommon site for a sore. Fig. 3 a large scar on the upper external surface of the thigh of a boy, and Fig. 4, two scars on the lower part of the chest of another boy.

It will be noted, among other things, from these photographs that though sores are most commonly found on the exposed parts, such as the face, legs, arms, they do sometimes occur on the scalp, chest and abdomen.

The figures on Plate LVI illustrate some interesting appearances seen in smears made by puncturing sores in various stages of development, and staining them with Romanowsky's stain. Fig. 1 shows a large macrophage containing 62 parasites from a smear taken from a recent sore. These large cells are sometimes seen so full of parasites, that there can be very little doubt that they rupture, portions of the protoplasm with a number of parasites in them breaking away. Fig. 2 shows another macrophage containing 49 parasites. In many of these cells it will be noted that the parasites are enlarged, the blepharoplast and the nucleus appearing to be on the point of dividing, so that there can be very little doubt that the parasites multiply in the cells and thus eventually lead to their bursting. Figs. 3, 4, 5 and 9 represent an appearance sometimes seen on the macrophages in young, actively growing sores before they become ulcerated. The parasites are enclosed in what appears to be a capsule suggesting a process of sporulation, but in reality the whole mass represents a part of the protoplasm of another macrophage which has ruptured, and the detached portion has been engulfed by another cell. Fig. 9 shows one of these sporulating masses as seen free in a smear from a sore; it is part of the protoplasm of a macrophage which has ruptured. Fig. 6 illustrates a single parasite in a polymorphonuclear leucocyte, and Fig. 7 another in a large mononuclear, both were recovered in a smear from the edge of a sore. Fig. 8 is a large macrophage showing degenerating parasites in its protoplasm. It will be noted that the outlines of most of the parasites have disappeared and only the nuclei and blepharoplasts can be seen, when stained with Romanowsky's stain. These cells are commonly seen in smears from sores which are about to heal up, showing that the parasites, having ceased to multiply, are now degenerating. Fig. 10 is a large mononuclear containing two parasites which was found in a film made from the peripheral blood of a patient with a young actively growing sore. Figs. 11, 14, and 15 illustrate polymorphonuclear leucocytes containing many parasites from the margin of a young sore. Figs. 13 and 16 illustrate two large mononuclears, one with a single parasite, the other with two, also from the margin of a young sore. It will be noted from these drawings that large numbers

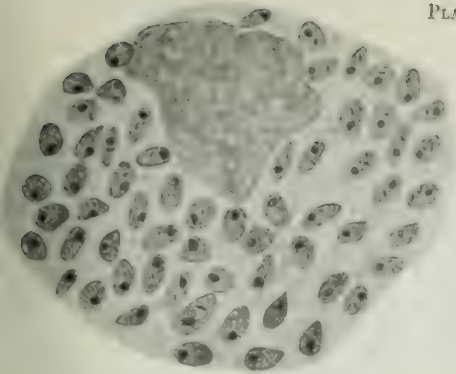


FIG. 1

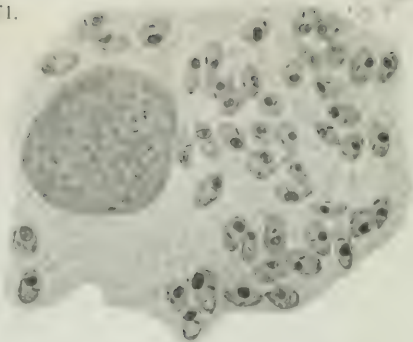


FIG. 2

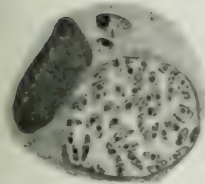


FIG. 3

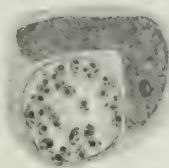


FIG. 4

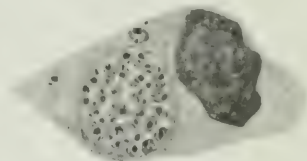


FIG. 5

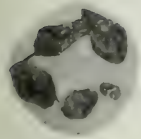


FIG. 6

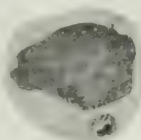


FIG. 7



FIG. 8

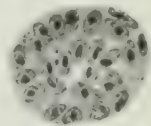


FIG. 9



FIG. 10

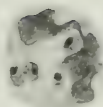


FIG. 11

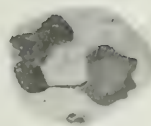


FIG. 12

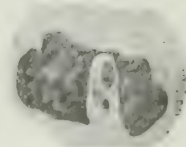


FIG. 13

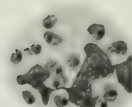


FIG. 14

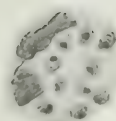


FIG. 15

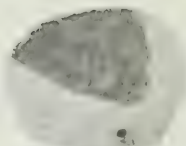


FIG. 16

*Herpetomonas tropica*.



of parasites may be picked up both by polymorphonuclear and large mononuclear leucocytes, and there seems no reason to doubt that these may find their way into the circulating blood. The fact that I have found the parasite in a film made from the peripheral blood supports this view. It would be interesting to culture the peripheral blood of a patient with one or more sores in an active stage on NNN medium to see to what extent parasite is carried into the blood. I believe, if this were done, frequently positive results would be obtained.

It is next necessary to consider how and from what source the parasite enters the human skin. And here again, as in the case of *Herpetomonas donovani*, we must start from the standpoint that the parasite is an insect flagellate belonging to the same genus, and that the flagellate stage is passed outside the human body. I do not for a moment believe that the parasite is transmitted from man to man by direct contact, thus excluding an insect phase in its life history, and more than nine months' residence in Cambay convinced me that this never occurs in nature. It is true a patient does re-infect himself by transferring some of the parasite-laden discharge on his nails from a sore to any small abrasion or scratch on another part of his body. This regularly takes place, and it is quite common to see a patient with multiple sores. This occurs quite apart from re-infection in the natural way, for I have had a case under my observation in Madras where Oriental Sore does not occur. This man was infected by me from my own sore in Madras, and had never been in Cambay or any other Oriental Sore locality. During the time his sore was discharging living parasites in the serous fluid exuding from it, he unconsciously transferred the parasites to other parts of his body when scratching himself. There could be no doubt as to the nature of the secondary sores, which were about twelve in number and scattered all over his body, because I found the parasite in smears from them.

If then we exclude infection from man to man by contact, a method of infection which I am convinced never happens in nature, we must assume that the parasite passes out of the human body, and finding itself in a suitable environment becomes a flagellate. Can the parasite be acquired by being ingested in food, etc.? This would assume that it passes through the wall of the stomach into the blood and then reaches the skin. This method of infection can, however, I am sure, be ignored without any further comment, as being impossible. We must then assume that the

parasite is either directly inoculated into the skin or deposited on it through the medium of an insect. Let us now consider the most probable way in which this may happen.

House flies such as *Musca nebulo*, *Musca humilis*, and the eye fly, *Siphunculina funicola* are attracted to the discharge from these sores, readily feeding on the serous fluid. In a suitable case this discharge is loaded with living parasites, and it will be readily understood that these insects would infect themselves by ingesting large numbers and also contaminate their legs, etc. In the case of *Musca nebulo*, I have shown that the parasite never flagellates in the flies' alimentary tract and never reaches the rectum in a living condition, so that it is difficult to understand how it could infect a clean cut. I endeavoured in Cambay to infect myself by allowing flies to settle on cuts, etc., after having walked over a suitable sore, but the cuts never developed into sores. I feel certain the same would happen in the case of the other two species mentioned. In the case of the eye fly, however, it should be noted that it is infected with a species of *Herpetomonas* described in this Journal. This parasite is clearly not connected with that of Oriental Sore, but is a distinct species. It is common in the eye fly in Madras yet Oriental Sore does not occur there. From my observations in Cambay, I came to the conclusion that as the parasite never flagellates in the alimentary tract of *Musca nebulo* and cannot even survive in it, that the house fly plays no part in the transmission of the parasite.

As there are no other insects which are likely to become infected directly from the sore by feeding on it, or the discharge from it, we must assume that the parasite may be sucked up by a blood-sucking insect. The most likely insects are as follows :—

1. Mosquitoes.
2. Sand Flies (*Phlebotomus*).
3. Lice.
4. Fleas.
5. Bugs.

*Mosquitoes*.—I have not experimented with any species of mosquito by feeding it on the blood of a patient, so am unable to say whether the parasite would develop into a flagellate in its alimentary tract. It is true Wenyon claims to have recovered the parasite of Oriental Sore in its flagellate stage in *Stegomyia* by feeding this mosquito on sores at Bagdad ; I have no doubt, however, that the flagellate seen by him is nothing more than a natural *Herpetomonad* of *Stegomyia*. The fact that

he never observed the round stages of the parasite of Oriental Sore developing into a flagellate in the midgut of the mosquito, stages which should have been seen if he were dealing with this parasite and not a natural one, supports my interpretation of his findings. The evidence in favour of the parasite being transmitted by a mosquito is not very convincing, and I believe these insects may be safely excluded as being the carriers of the parasite of Oriental Sore in India.

*Sand Flies (Phlebotomus).*—The sand fly has been incriminated as a carrier of the parasite by a number of observers in different parts of the world, mainly on epidemiological grounds. As is well known these flies bite on the exposed parts of the body, where Oriental Sores nearly always develop. No one has, as far as I am aware yet, been able to demonstrate that the parasite will develop into a flagellate in the midgut of the sand fly. As the possibility of the sand fly ingesting the parasite in the circulating blood of a patient with a sore was considered remote, it was necessary to explain how it becomes infected. It is well known that these flies feed on the blood of house lizards belonging to the Family Gekoniæ, and here was a possible source from which they might acquire the parasite. This view has led to a number of observations on the Gekoniæ as the possible reservoirs of the parasite. A species of *Herpetomonas* has been found in the organs of the house gecko, *Tarentola mauritanica*, by a number of French observers in North Africa, and the conclusion was drawn that this gecko was in all probability the reservoir of the parasite of Oriental Sore in that country, much in the same way as many antelopes are the reservoir of *Trypanosoma gambiense*. Such an hypothesis would at first sight appear to be a very suggestive one, but unfortunately no one has yet succeeded in finding this *Herpetomonas* in the peripheral blood of this gecko; and I note with interest that Nicolle has now abandoned this hypothesis, and believes the gecko can play no part in spreading the parasite. The possibility of the sand fly itself being the reservoir of the parasite of Oriental Sore cannot be ignored, as I will now point out. In Mesopotamia, where Oriental Sore is very common and widely distributed, the sand fly seemed to me the only possible insect which could be the host of the parasite. I have already pointed out that two species of *Phlebotomus*, *P. papatasi* and *P. minutus* are known to be naturally infected with species of *Herpetomonas*, *H. phlebotomi* v. *P. minutus* in India (Assam and not Madras), and *H. sp. ?* in *P. papatasi* in Palestine and probably in Mesopotamia. Can these species of *Herpetomonas* themselves be but the insect phase of the parasites of Oriental Sore in

India and Mesopotamia, and if so, how do they become pathogenic to man? I believe that the parasite of Oriental Sore in Mesopotamia is the vertebrate phase of the *Herpetomonas* of *Phlebotomus papatasi*, and that it penetrates into the skin of man when an infected *Phlebotomus* is crushed on the skin. Any one who has been in that country will know that during the sand fly season the fly is daily crushed on the skin, when it is in the act of biting. Owing to the small size of the fly, it is often overlooked, but the immediate stinging sensation caused by the bite directs the victim to the spot and the fly is crushed almost every time; once the sand fly has begun to suck blood it is not readily disturbed until it has become replete. At present this is only a hypothesis, but it is one which lends itself to easy proof, and I hope this will be done. Some support for this explanation of the method by which the parasite of Oriental Sore is acquired has been given by a recent experiment recorded by Laveran and Franchini. These observers have succeeded in infecting an adult dog with a local lesion by inoculating it with the *Herpetomonas* of *Phlebotomus papatasi*; the parasite was recovered in the cells in the lesion and was exactly identical with that of the well-known parasite of Oriental Sore in man. I consider a similar experiment should be carried out in the case of man.

Is the sand fly in India the reservoir of the parasite of Indian Oriental Sore? I am unable to express any opinion on this question for the reason that no species of *Phlebotomus* has been found to be infected with *Herpetomonas* in that part of India where Oriental Sore occurs. In Cambay the sand fly, *Phlebotomus minutus*, was scarce and difficult to obtain, so that I was not able to make any observations on it. So far as I was able to discover it is absent in Cambay during the cold weather, from November to February, when the parasite of Oriental Sore is acquired, so that it would appear to be an unlikely source of the parasite. It is interesting to note that though according to Mackie 10 per cent of *Phlebotomus minutus* are infected with *Herpetomonas phlebotomi* in Assam, Oriental Sore is not endemic in that country. It may be that temperature conditions are unsuitable to the propagation of the parasite, or it may be that this species of *Herpetomonas* is not one which has yet become pathogenic to man. Perhaps after all the species found in *Phlebotomus papatasi* is the only one which can live in the human skin. Experiments in this direction in India would unquestionably lead to some valuable and interesting results.

*Lice.* I am convinced that lice do not play any part in the transmission of the parasite of Oriental Sore in India, for all my observations at Cambay in this direction were negative.

*Fleas.*—The same may be said regarding fleas. There is no evidence that any species of flea is the invertebrate host of the parasite. I consider that any experiments with these insects would be a waste of time.

*Bugs.*—*Cimex hemiptera* is the only species of bug which is likely to be concerned in the transmission of the parasite in Cambay. It will be remembered that by feeding this species on the peripheral blood in the neighbourhood of a sore, I was able to observe the development of the parasite from the unchanged condition in a leucocyte into a typical flagellate; this change only takes place in the midgut of the bug at a temperature below 25°C. It is a striking fact that Oriental Sore only occurs in those parts of India where there is a decided cold weather, the temperature for several months of the year being well below 25°C. The fact that the parasite will flagellate in *Cimex hemiptera*, but only under a certain temperature, and that this temperature corresponds to the distribution of the parasite in India, is in my opinion very strong evidence that the bug is the invertebrate host of *H. tropica*.

In another paper in this Journal I have described with LaFrenais and Sundara Rao the change undergone by the parasite in the alimentary tract of the *Cimex hemiptera* as seen in stained smears of the contents on successive days after a feed on flagellates. From these appearances it was concluded that the parasite rounds up towards the end of a feed, and that it tends to disappear after a time. In a nymph fed six days after the original feed, it was found that there were many rounded young flagellates multiplying in the fresh blood, thus producing an intense infection of the midgut. But here as in the case of the parasite of Kala-azar it was realised that the examination of the contents of the alimentary tracts of bugs, both in the fresh and stained condition, in the first place would not be likely to lead to any satisfactory results, and instead the different parts of the alimentary tracts of bugs which had fed on flagellates were cultured. As in the case of the parasite of Kala-azar it was possible to find out how long the parasite could live in the alimentary tract of the bug.

In adult bugs fed on young cultures of *Herpetomonas tropica*, and not refed on human blood, the parasite was recovered on the 23rd day from the various parts of the alimentary tract of a bug; nymphs and larvae were not experimented with. In the case of bugs fed on flagellates and

then refed at intervals on clean, human blood the following results were obtained. A positive culture was obtained from the contents of the midgut and rectum of an adult bug 34 days after the infected feed, and 44 days from the contents of the hindgut. A positive result was obtained from the contents of the midgut of a nymph 31 days after the infected feed, and from the contents of the hindgut and rectum 36 days after the infected feed. In the case of the larvæ a positive result was obtained on the 9th day after the original feed, the bug having fed as a larva. From these results it will be noted that the parasite can live as long as 44 days in the hindgut of the bug, a result which is very similar to that of the parasite of Kala-azar.

As soon as I was able to confirm Mrs. Adie's discovery of an intracellular stage of *H. donovani* in the cells of the midgut of *C. hemiptera*, it occurred to me that a similar method of development might take place in the case of *Herpetomonas tropica*. And there seemed every reason to believe this, for Cornwall has shown that this parasite also becomes a thick tail in the alimentary tract of *Cimex*, and not in the midguts of several blood-sucking insects he experimented with; and I have myself seen this stage in the alimentary tract of *Cimex hemiptera* after a feed of the flagellates of *H. tropica*. The technique adopted for the study of the intracellular stage of *H. donovani* was at once tested in the case of *H. tropica* with excellent results. In this way it was possible to observe the intracellular stage. The flagellates here also, on entering a cell, develop a sheath and then curling up become round; the movements of the flagellum within the sheath produce the characteristic thick tail. The encapsuled flagellate, while coiled up in its capsule, divides and several daughter flagellates are produced, which in time leave the cell by the rupture of the capsule; it is common to find a number of empty capsules lying about in a fresh preparation. Although I have not observed a flagellate of *H. tropica* actually pass into a cell, the examination of many fresh and stained midguts have left no doubt of the existence of this intracellular multiplication. I hope on another occasion to take up the study of this stage and to cut sections of infected midguts.

From these observations it is now clearly established that both *H. donovani* and *H. tropica* behave in exactly the same way in the midgut of *Cimex hemiptera*. I have little doubt that many of the forms I have illustrated in another paper with LaFrenais and Sundara Rao on the behaviour of this parasite in the alimentary tract of the bug, represent further, and perhaps the final, stage in the development in the bug.

How is the parasite transmitted to man by the bug? After infecting bugs fed on rich cultures of *Herpetomonas tropica*, I have searched in vain in the salivary glands for parasites, so that it is difficult to understand how the parasite could be transmitted by the bite of the bug. Further, I have fed large numbers of infected bugs on the human subject also with negative results, so that I think this method of infection may be safely excluded. The only other way an infected bug can convey the infection is when it is crushed on the skin. In my preliminary report on the development of the parasite in the bed bug, I drew attention to two cases in which sores developed at the sites where bugs were crushed.

Before it is possible to carry out transmission experiments with bugs by crushing them on the human skin, it would be advisable to discover the infective stage in the bug, and more particularly, when and where it is likely to be found. The fact that the parasite of Oriental Sore can be readily transmitted to man by placing some of the parasite-laden serous fluid from a sore on a slight abrasion on the human skin suggests that the parasite is usually transmitted in nature in a round stage. There is no doubt that the discovery of the infective stage in the bug in the case of *H. tropica* would give us the clue to the similar stage of *H. donovani*, especially when we know that both parasites behave in the same way in the midgut of *Cimex hemiptera*.

Thanks to Mrs. Adie's brilliant discovery of the intracellular stage of *Herpetomonas donovani* in *Cimex*, these two problems are now nearing solution, and I have little doubt that before long many of the puzzling phenomena connected with the spread of these two human diseases in nature will be cleared up. It is now nearly 16 years ago that I first saw the flagellate stage of *H. donovani* in *Cimex hemiptera* fed on the peripheral blood of a case of Kala-azar. Since then I have held the opinion that the flagellate seen on April 2nd, 1905, represented a true development in a natural insect host; and have continued my work in spite of severe criticism, believing that some day convincing evidence would be brought to light. This has now come in an unexpected way. And it is pleasing to know that the last links in the chain of evidence incriminating the bug as the carrier of the parasite of Kala-azar have been forged by two well-known laboratory workers in India.

In conclusion I wish to take this opportunity of thanking Sub-Assistant Surgeon Sundara Rao of the Pasteur Institute of Southern India for the valuable help he has given me in many directions, and more particularly in the delicate manipulations required when culturing the

various parts of the alimentary tract of the bug. And when I say we have cultured 1,233 bugs the arduous nature of the task will perhaps be appreciated by those who can realize what this means. Like myself, he has never hesitated to feed large numbers of bugs heavily infected with *H. donovani* on himself, so that our results may be similar to what happens in nature. It remains to be seen whether we develop Kala-azar, but so far no local lesions have developed at the sites of the bites of the bugs.

I wish also to thank Mrs. Adie for coming to Coonoor, and showing me her work on the intracellular stage of the parasite of Kala-azar. I must confess I hesitated to believe in its existence until I had seen it myself. I wish also to thank Colonel Cornwall, I.M.S., for his help and advice in studying the intracellular stage in the case of both parasites.

# A CONTRIBUTION TO THE STUDY OF KALA-AZAR (V).

BY

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[Received for publication, July 26, 1921.]

IN 1915-1917, a series of papers from this laboratory was published in this Journal dealing mainly with the development of *Leishmania donovani* and *L. tropica* in the stomach and gut of the bed bug. A new form of the parasites, described provisionally as a "thick-tail," was noted, and serial sections of the stomach and gut of bugs fed on cultures of *L. donovani* and of *L. tropica* were cut and examined for an intracellular stage, but no evidence of it could be found. The role of the "thick-tail" remained obscure.

The subject was then dropped in favour of other work.

Recently Mrs. Adie(2) has announced that she has been able to follow the development of an intracellular stage of *L. donovani* in bugs fed on spleen juice obtained from infected persons.

As a result of discussions with Mrs. Adie, whose findings differed so widely from our own, we determined to repeat some of our previous work particularly the sectioning of the stomach and gut of infected bugs.

*Method.*—Bugs were fed through a rabbit skin membrane on cultures of *L. donovani* and of *L. tropica* mixed with extracted material

blood. Some were kept at 20°—22°C, others at 27°C. As the experiments proceeded the weather grew warmer and the laboratory temperature rose and remained between the limits of 24° and 27°C, usually about 25·5°C. The fed bugs were then kept in the dark at laboratory temperature. Beginning at 16 hours after the feed, and thereafter daily, bugs were dissected and their stomach and gut removed. Some were dropped into a fixative, others were smeared out on slides and others were examined at once in the fresh state in saline under a vaselined cover. Trials were made with many fixatives and several stains, but we found that we could not improve on the technique employed by Minchin and Thomson(3) in their investigation of the development of *Trypanosoma lewisi* in the rat flea, so that was more or less closely adhered to. Tissues were fixed in Maier's modification of Schaudinn's fluid and stained with Romanowsky's mixture and differentiated in acetone-xytol. Others were stained with Heidenhain's iron hamatoxylin. Minchin and Thomson dwell on some of the disadvantages of their methods and we fully agree that they are by no means ideal. Nevertheless the results obtained by the Romanowsky staining are generally good enough to show up the parasites clearly in the stomach contents, though not well in the black contents of the gut, and intracellular development could not be passed over.

Another method tried was to suspend the excised stomach or gut of starved bugs in a fluid containing numerous active flagellates in a narrow pipette. After a varying period of hours the viscus was expelled into a fixative and in due course sectioned. The staining properties, however, of the cells became altered by post mortem and digestive changes and good results could not be obtained, so this method was soon abandoned.

*Material.*—Feeding experiments made :—

1. February 16th, 1921. Bugs were fed on a culture of *Leishmania donovani* mixed with citrated rabbit's blood.

Lot A. Fed and kept at laboratory temperature 19°—20°C till dissected.

Lot B. Fed and kept at 27°C till dissected.

2. February 28th. Bugs were fed as above on *Leishmania tropica* cultures.

Lot C. kept at 20°C.

Lot D. kept at 27°C.

3. April 25th. Bugs were fed on a freshly-excised, pounded-up Oriental Sore mixed with citrated human blood.  
Lot O. S. kept at laboratory temperature, about 23°C.
4. May 5th. Bugs were fed on *L. tropica* culture mixed with citrated rabbit blood.  
Lot L. kept at laboratory temperature, about 24°C.
5. May 29th. Bugs were fed on *L. donovani* culture mixed with citrated human blood.  
Lot E kept at laboratory temperature 24°-27°C.

The serial sections of the stomach gave about 200 sections of each specimen when the organ was distended with blood, fewer as the blood became absorbed and the organ contracted.

The guts, being so small and tenuous, could not be stretched and cut from end to end: they were embedded in the coiled form they had assumed and the coils were cut at hazard as if a solid piece of tissue was being dealt with.

In the 5 series about 120 bugs were examined in the fresh state and sections were cut of the stomachs and guts of about 120 more, so some 20,000 sections of stomachs and a like number of guts were available for scrutiny. The sections were all cut by Laboratory Assistant M. K. Motha.

#### THE EXAMINATION OF FRESH SPECIMENS.

It is by no means easy to remove from a bug a stomach distended with blood without rupturing it. Laboratory Assistant M. B. Ajjan acquired considerable skill in the process. The unruptured stomach in a drop of saline on a slide is covered with a slip thickly vaselined at the edges, so that it will not press on the specimen. The slightly flattened stomach then can be examined conveniently with an oil immersion lens, and with powerful artificial light the cells all round the optical section are clearly outlined. Moving objects in the stomach contents can also be seen. In no instance could parasites in any stage be detected within cells, although they could be seen clearly moving about in a free stage in the stomach contents. The activity of flagellates in the stomach contents bears an inverse relation to the viscosity of the contents.

After the examination the unruptured stomach has revealed all it can, the vaselined cover glass is gently pressed till the stomach wall is split, or it may be lifted and the stomach cut up with sharp

needles. A large number of the epithelial cells can then be examined in detail.

Another method is to dissect out the stomachs of starving bugs and float them in a flagellate culture either in a pipette or under a vaselined cover glass. If the preparations are kept sterile they last for 48 hours and the relations of the parasites to the cells can be observed at any time. We have often observed in fresh preparations such as these, not only flagellates, but the encysted, wriggling, round bodies, which are derived from thick-tails, lying apparently within cells; but in preparations that have been thus manipulated one can hardly say for certain that these were undamaged cells.

After a full feed the stomach may be so distended that the greater part of the epithelium is flattened, and there seems to be insufficient room in these flattened cells to accommodate proliferating parasites. There are, however, generally some patches in the stomach where the epithelium escapes this flattening by distention and the cells retain their cylindrical shape. These cells have large, oval or rounded nuclei and bulge into the lumen of the stomach. They may contain globules of a clear fluid with dancing granules. The bulging part of these cells melts into and mingles with the stomach contents. A cell discharges its secretion, reloads and again discharges probably many times before it becomes finally exhausted; but at length the whole remains of the exhausted cell, including the nucleus, is exfoliated. Points where regeneration of the epithelium is proceeding are frequently seen consisting of several small dividing cells in close apposition. These, by the way, might readily be mistaken by a careless observer for multiplying bunches of parasites.

#### THE EXAMINATION OF SMEARS.

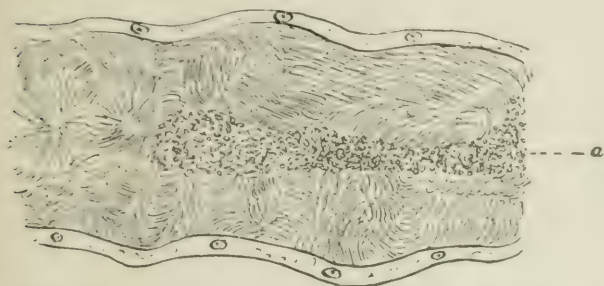
It may be said at once that the examination of the smears stained by Romanowsky's method brought to light nothing definite as regards an intracellular phase.

#### GENERAL REMARKS.

In the stomach of some bugs the flagellates do not multiply freely but die out altogether within a period of days. In other bugs, multiplication is free and the bug remains infected for a long period.

In a small proportion the multiplication of the flagellates is incredibly great, not only the stomach, but also the upper part of the gut become mere bags filled with a seething mass of the organisms. (V Fig. 1.) A bug thus infected refuses food and often dies, whether from intoxication or the mechanical action of the parasites is not known. The flagellates may be found alive in the gut 24 hours after the death of the bug.

FIG. 1.



A portion of the unopened gut of a bug fed on *L. donovani* cultures. Seen in the fresh state in optical section.

(a) is a plug of granular food-debris. Surrounding it are masses of multiplying flagellates, so closely packed that movement is impossible until they are set free.

As a rule unaltered erythrocytes do not find their way from the stomach to the gut, the stomach is the digestive part of the alimentary tract and the gut is the absorptive part, so the stomach contents are not passed on until the preliminary proteolytic processes have been completed. Occasionally, however, after a very full feed a few intact erythrocytes get carried over into the gut almost immediately. It is possible therefore for flagellates to be carried into the gut with them and so the infection of the stomach and of the gut may be almost simultaneous. When a bug has not over-distended itself and no intact erythrocytes have reached the gut, one may usually count on finding a considerable number of flagellates in the gut within 16 hours of the infected feed. It is not known whether the reaction of the stomach differs from that of the gut; anyhow the cavities seem to be equally favourable to the multiplication of the flagellates as regards numbers.

As regards morphology there seems to be little difference, though the prevailing stage present at any one moment may not be the same in both stomach and gut.

When a bug sucks in a feed containing a culture of flagellates it generally gets a sample of each of the different forms present in a culture, from the round body with no flagellum to the fully developed flagellate. We cannot pretend to say exactly what happens to each of these forms when it finds itself in the stomach of a bug, nor does it seem to us a matter of much moment, at least just now, but it is easy to give a general idea.

*In the stomach* at first the rounded and semi-developed flagellates prevail, and large clumps of them can be seen; later, these develop into active flagellates which further subdivide and swim freely. (*vide* Fig 2.) Even when free swimming flagellates seem to be in a majority there are always plenty of rounded and half developed forms also. As the food supply in the stomach gets digested and passed on into the gut, the parasites either pass on with it or die off; and when the stomach seems to contain nothing but air bubbles it may require some search to find a few sluggish flagellates or rounded forms, generally none can be found. A fresh, sterile feed may or may not revive the remaining parasites and start a fresh brood.

For example, some bugs were fed on *L. tropica* cultures mixed with citrated rabbit blood on February 28th, 1921, at 27°C and maintained thereafter at that temperature. They were refed naturally on a normal rabbit on March 5th, 11th, 15th, 19th, 24th and 29th. Five were dissected on March 31st. No parasites were found in the stomach or gut of four, but in the fifth in the stomach there were enormous numbers of free adults and huge dividing clumps, and in the gut vast numbers of adults and rounded forms.

*In the gut* the long, free, active flagellates appear in huge numbers a little later than in the stomach, so that in one and the same bug one may find the stomach nearly empty and containing comparatively few parasites while the gut contains multitudes of active flagellates.

No particular experiments have been made to find how long the gut can contain living flagellates after the stomach has cleared itself nor whether parasites would ascend from an infected gut after a fresh feed and re-infect a stomach which had become free from them.

In a previous paper of this series we have shown that living flagellates seldom descend the alimentary tract as far as the rectum.

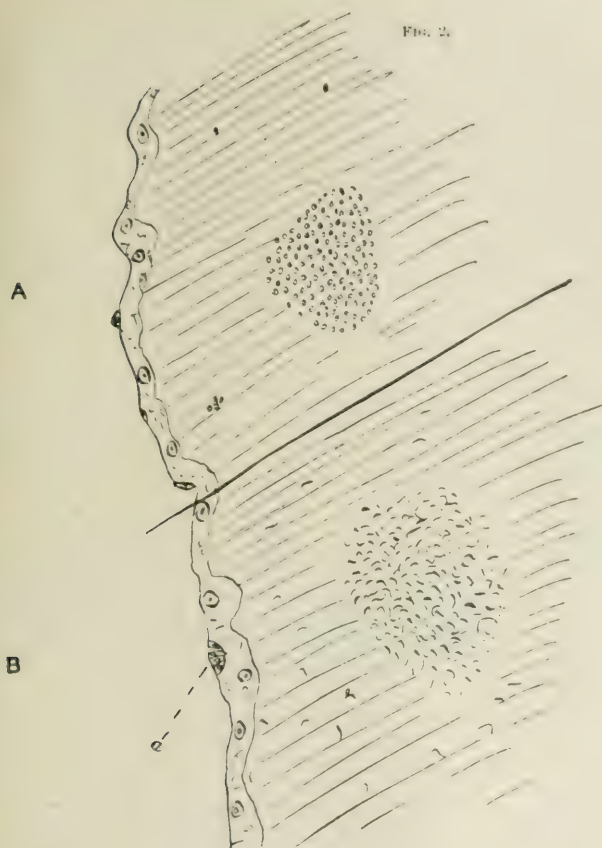


FIG. 2.

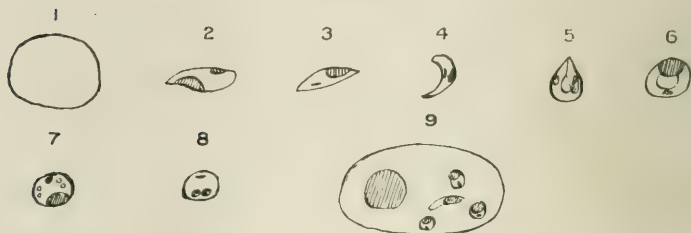
Appearance of a section of the stomach of a bug fed on cultures either of *L. donovani* or of *L. tropica*, stained with Romanowsky.

- A. Shows a clump of dividing, non-flagellated spheres lying in the stomach contents with a few scattered individuals.
- B. Shows a similar section with a clump of flagellates and free flagellates scattered about in the stomach contents. Note that in neither instance has the mass of multiplying organisms any relation to the epithelium.
- (a) Is a clump of young epithelial cells.

The following case is of some interest :—

An "Oriental Sore," acquired in Rajputana, which was still covered with intact epidermis, was excised after injecting round it cocaine and adrenalin. Half the nodule was cut up and pounded in a mortar and the pulp was mixed with defibrinated human blood. Bugs were fed on this mixture through a rabbit skin membrane. For some reason, perhaps because the drugs tasted unpleasant, the bugs after a trial mostly re-used to feed. A fair number, however, had sucked in a sufficiency of the fluid. Numerous flagellates were found in the stomach 42 hours after the feed and in the gut 64 hours after the feed. The bugs had been kept at laboratory temperature, about 23°C. (*Vide* Figs. 3 and 4.)

FIG. 3.



Smear from a non-ulcerated Oriental Sore.

1. Erythrocyte for size comparison.
- 2-8. Forms of *L. tropica* found free in the smear.
9. An endothelial cell containing four parasites.

FIG. 4.



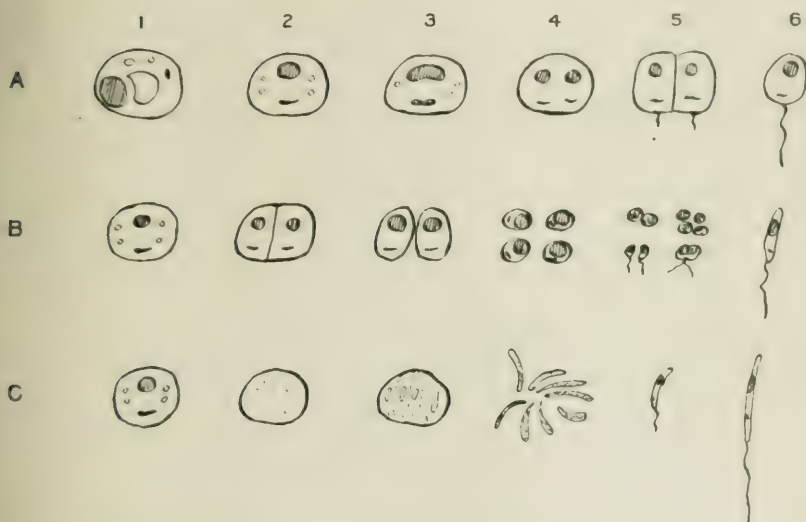
Flagellated forms of *L. tropica* from the stomach of a bug 42 hours after feeding on material taken from the Oriental Sore.

Generally speaking the only difference noticed in the march of events when one lot of infected bugs is kept at, say, 20°-22°C and another a

27°C, is that digestion proceeds much more rapidly at the higher temperature, the stomach empties itself of food more quickly and consequently the parasites die out sooner.

The developmental forms seen are the same under both conditions, whether *L. donovani* or *L. tropica* be the parasite experimented with.

FIG. 5



Diagrammatic representation of three modes of development of flagellated bodies from spheres.

*Thick-tails*.—A word must be said about the "thick-tail" form. This is clearly an encapsuled flagellate. Occasionally two flagellates may be seen enclosed in the same capsule, having arisen presumably by division of the first. Division may even proceed till there are four flagellates within the capsule, but of this we cannot be certain. It is very difficult to follow the contortions of the bodies within the capsule in the living state and the capsule cannot be stained, so that in a smear one cannot say whether one is looking at an ordinary bunch of flagellates or at an encapsuled form. We have got but little further in explaining the

origin or the termination of the thick-tail than we reached in 1917. All we know is that it does not appear in cultures and that close contact with the epithelium of the stomach or the hind gut of the bed bug appears to be necessary for its appearance. The guts of other blood-sucking insects so far tried appear not to produce this form. Thick-tails cannot be found in every bug, and when found there are often 20 or 30 of them very close together, as if a particular portion of the epithelium had favoured their appearance.

It may be concluded with confidence that the thick-tail does not form an essential link in the chain of development of a perfect flagellate in the stomach or gut of a bed-bug, for many thousands of flagellates may be found for every thick-tail. Although one or more perfect flagellates may emerge from a thick-tail, yet its appearance in the bug is to some extent accidental, and we do not know whether it is a state assumed to protect against a harmful environment or whether it is a phase in the normal development of the parasite which is rendered uncommon, or aborted, by unfavourable conditions in the stomach of the bug, but which would be a common, if not an invariably necessary phase, if the parasite were to find itself in its real host.

As we have no evidence to show that the thick-tail is a protected form, which enables the parasite to tide over temporarily unfavourable conditions in the bug and to continue its developmental career when favourable conditions are established, we are more inclined to the hypothesis that it is a necessary phase which will only find its full explanation when the true insect hosts of the parasites are discovered or the extra-human life histories of *L. donovani* and of *L. tropica* are worked out.

#### ARGUMENT.

Active flagellates may easily penetrate into damaged cells; indeed, it is difficult to say what is a damaged and what is an undamaged cell, for the healthy, undamaged, actively secreting cells which are throwing off globules and cell substance are practically open to the invader, and flagellates may penetrate as much by accident as by chemiotaxis. It may be that this is the explanation of the thick-tail, that a flagellate having penetrated into a secreting cell finds itself in an unfavourable medium and promptly surrounds itself with a protecting membrane within which it is secure and may even divide. Later, when thrown off in globules

of secretion it finds itself in more congenial surroundings, bursts its capsule and resumes the free life of a flagellate.

On no occasion, from 16 hours to 10 days and even longer after the infecting feed, have we seen any other kind of apparently intracellular parasite in any stage of development, and we feel that if these flagellates actually enter d cells in the stomach or gut of the bug in the course of their developmental cycle and divided there in the manner described by Minchin and Thomson as occurring in the cycle of *Trypanosoma lewisi*, we could hardly have failed to observe them, considering the large amount of material we have worked over.

In none of the hundreds of stained sections of the stomach and gut that we have examined could we find any evidence of an intracellular stage of development, though it is true that on a few occasions we saw in cells single bodies which were indistinguishable from forms of the parasite. They may have been parasites in the cells or they may have been artefacts. Anyhow the proverb tells us that 'one swallow does not make a summer.'

Neither in fresh preparations nor in sections does it appear that the epithelium has an overwhelming attraction for flagellates. In fresh preparations as many flagellates can be found in outlying parts of the preparation as in the neighbourhood of the epithelial cells; in a section the parasites seen to be fairly evenly distributed throughout the contents of the viscus, large clumps may be seen close to the epithelium but equally large clumps may be found in the middle of the lumen.

Patton has described the development in the stomach of a bug of flagellates from the round, Leishman-donovan bodies contained in leucocytes of the blood sucked from a Kala-azar patient. If flagellates can develop directly from the Leishman-donovan body, there is no need to postulate the existence of a minuter phase of the parasite in human peripheral blood which will develop into a flagellate before or after entering a cell in the stomach of a bug.

Minchin and Thomson state that the free, active, rat *Trypanosoma* do not undergo any form of multiplication in the stomach contents of the flea, and that the only multiplicative phase is intracellular.

*L. donovani* and *L. tropica*, on the other hand, multiply freely by division in the stomach contents of the bug. It does not seem to be probable that two kinds of multiplication would go on simultaneously in the same viscus, the one intracellular and the other extracellular. There may, however, be instances of this happening which are unknown to me.

At present we feel that as the flagellate can multiply so freely in the contents of the stomach and gut of the bug there is no need for an intracellular phase ; but the mere facts of flagellation and multiplication do not suggest an explanation, in the circumstances, of the use of a flagellum to the parasite nor the use of free multiplication in promoting the continuity of its life cycle.

If an obligatory intracellular phase were to occur it would form strong evidence in favour of the bug being the true insect vector of Indian Leishmaniasis ; if it does not occur, then there is no reason to regard the bug as any thing more than a casual host in which development of the parasite can proceed to a certain point but no further.

We have stated before that we recognize that our experiments have not been quite natural ; we have fed our bugs on cultures and not on infected persons or animals, and it may be argued that the results are invalidated thereby. We are, nevertheless, ourselves persuaded.

The Oriental Sore feeding experiment was nearer nature, though not quite natural, and that too gave no positive intra-cellular development. If we might venture to offer counsel, it would be that the bed bug be given a rest in connection with Leishmaniasis and that some other possible vector be carefully investigated.

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## NOTE ON THE HISTO-PATHOLOGY OF A NON-ULCERATED ORIENTAL SORE.

BY

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[Received for publication, June 30, 1921.]

TITMOUTH CUNNINGHAM (1), who, in 1884, first noted the presence of a parasite in cells of the dermis in an Oriental Sore, and JAMES (2) in 1905 both described sections which they had made, it seems that there is still room for a little clearer understanding of the lesion. The present description is based on sections cut from an Oriental Sore which was excised with local anæsthesia. The sore was acquired by residence in Rajputana, though it was not noticed by its bearer, a European lady, till about three months after she had left that province to reside in the Nilgiri Hills where such sores are unknown to arise. It was situated over the ulceration process and was first noticed as a pimple. Attention was called to the spot by a sudden painful prick which she thought had been inflicted by a biting fly, though no fly was seen. This happened in the open air. The pimple slowly enlarged and several months later caused considerable pain and annoyance. When excised, it was about 1.5 cm. in diameter with a smooth, shiny, non-ulcerated surface. There were two other pimples each 3 mm. in diameter about 0.75 cm. distant from the main lesion, on opposite sides and separated from it by apparently healthy skin.

The main lesion was highly vascular and blood oozed freely from a capillary puncture made at its margin for diagnosis. Vast numbers of Leishman-donovan bodies of various forms were found in the smear.

The material was fixed in Zenker's fluid, Ehl's fluid and in a picro-formol-sublimite-acetic mixture and stained by various methods.

In the formation of an Oriental Sore the course of events is probably as follows : -the parasite is introduced into the dermis by way of a solution of continuity of the epidermis. It is improbable that it can establish itself at all unless it be deposited in the dermis : for not a single cell of the epidermis contained a parasite, not even the prickle cells of the rete mucosum, and it seems likely that the parasite would perish if there were any intact layers of the epidermis beneath the point of its entry. Parasites are not found in the cells of the sweat glands, sebaceous glands or hair follicles, probably because these are all developed from the malpighian layer of the epidermis. The mononuclear, endothelial cells found in the papillary layer of the dermis are the breeding grounds of the parasite and its establishment in the dermis appears to cause them to proliferate. The endothelial cells push their way in all directions following supposedly the lines of least resistance, that is mainly towards the surface of the skin.

The fibrous tissue of the dermis is split apart by columns of invading cells and becomes a mere network. New capillaries are formed to meet the requirements of the new tissue. At the same time hair follicles and sweat glands are gradually destroyed by pressure and by interference with their blood and nerve supply. The parasites meanwhile lag much behind the invading front of the endothelial cells, whether it be that they cannot multiply fast enough to keep pace with them or whether it be that the older cells in the middle of the mass are more suited to their nutritional needs. When the endothelial mass reaches up to the malpighian layer of the epidermis the nutrition of the whole thickness of the epidermis is affected, the dentate cells and many of the prickle cells disappear, the stratum granulosum cannot be distinguished, and the epidermis becomes reduced to a few layers of horizontally elongated, nucleated cells covered by a thin stratum corneum and surmounted by a stratum disjunctum. A little more time or a very slight injury will now suffice to remove some of this thinned, protecting epidermis and admit pyogenic organisms to a favourable nidus in the nodule, and in the process of ulceration which follows the protozoa are destroyed. Ulceration, therefore, is a curative process, though perhaps a crude and painful one. If it were not to occur, the parasites would spread laterally and destroy large areas of the integument.

In the normal dermis besides the endothelial cells there are plasma cells, migratory cells, mast cells and perhaps yet others. Some of these

take part in the struggle against the parasite. The endothelial cell is distinguished by a large, oval, faintly staining nucleus with a conspicuous nucleolus, and faintly staining cytoplasm. On the whole it resembles a nerve cell from a ganglion. When the cell is parasitized the nucleus becomes shrunken and shapeless and eventually disappears altogether and the cell seems to be replaced by a bag of parasites. Mingled with the endothelial cells, in a minority in the heavily parasitized area but in a great majority towards the periphery of the lesion, are smaller cells with a round, deeply staining nucleus. Some of these are plasma cells, others migratory cells. No extravascular polynuclear leucocytes are seen. The parasites do not appear to penetrate any of these cells or if they do, they are quickly altered and cannot be stained.

Presumably this collection of cells outside the parasitized area has some influence in limiting its extension both internally and laterally. When metastatic nodules arise beyond the area of the main lesion they are due to parasites being shed into lymphatic spaces and being carried outside the area before they are arrested.

Eosinophilous cells with large granules are entirely absent.

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# SOME NOTES ON INDIAN CALLIPHORINÆ.

## PART II.

LUCILIA ARGYRICEPHALA MACQ. (SERENISSIMA FABR.),\*  
THE COMMON INDIAN BAZAAR GREEN BOTTLE,  
WHOSE LARVÆ OCCASIONALLY CAUSE  
CUTANEOUS MYIASIS IN ANIMALS AND  
LUCILIA CRAGGII SP. NOV., ONE  
OF THE COMMON BLOW FLIES OF  
INDIAN HILL  
STATIONS.

BY

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ILLUSTRATED

BY

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[Received for publication, January 13, 1921.]

In the first paper<sup>1</sup> of this series I described *Chrysomyia bezziana* Vill., the important specific myiasis-producing Calliphorine of Africa, India, Burma, Assam and Ceylon, and at the same time recorded a number of cases of myiasis in man and animals caused by its larvæ. In

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\* Dr. Villeneuve informs me that *Lucilia serenissima* Fabr. is identical with *Lucilia argyricephala* Macq.



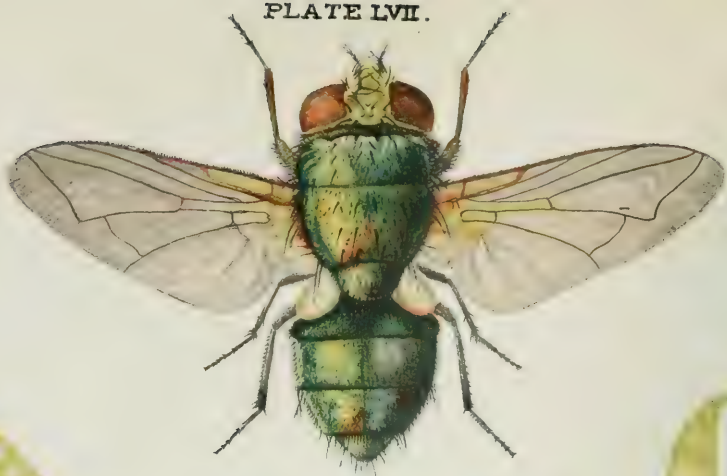


FIG. 4.



FIG. 1.



FIG. 3.



FIG. 7.



FIG. 5.

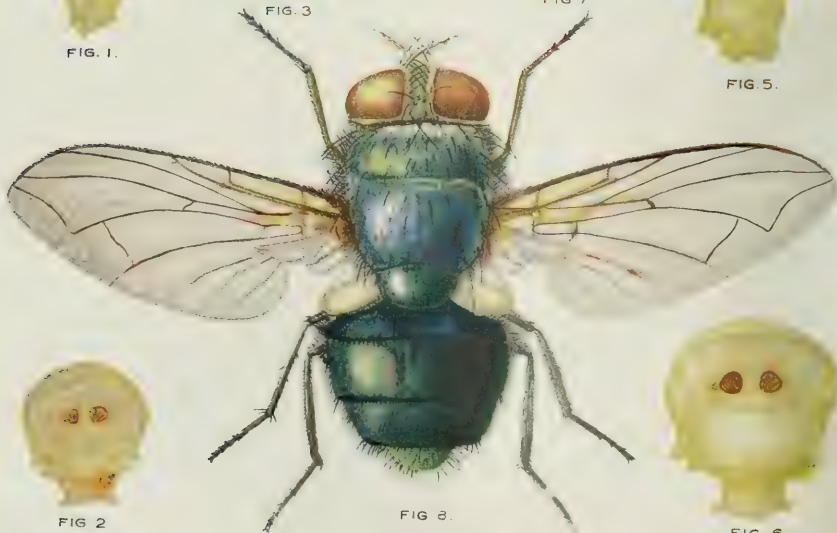


FIG. 8.



FIG. 2.



FIG. 6.

1871

Fig. 1. *Enallagma cyathigerum* ♂ & ♀.

Fig. 2. *Enallagma cyathigerum* ♂ & ♀.

Fig. 3. *Enallagma cyathigerum* ♂ & ♀.

Fig. 4. *Enallagma cyathigerum* ♂ & ♀.

Fig. 5. *Enallagma cyathigerum* ♂ & ♀.

Fig. 6. *Enallagma cyathigerum* ♂ & ♀.

Fig. 4. *Lucilia argyrocephala* ♀ × 3.

Fig. 1. Mature  
larva of  
*L. argyrocephala* : 5.

Fig. 3. Puparium of  
*L. argyrocephala* × 6.

Fig. 7. Puparium  
of *L. craggii* × 6.

Fig. 5. Mature  
larva of  
*L. craggii* : 6.

Fig. 8. *Lucilia craggii* ♀ × 5.

Fig. 2. End of larva of  
*L. argyrocephala* × 12.

Fig. 6. End of larva × 12.

the present paper, I propose describing another calliphorine, *Lucilia argyricephala* Macq., whose larvæ occasionally cause cutaneous myiasis in animals in India, and also a new species of *Lucilia*, one of the common blow flies of the hill stations in India.

Although I have received dipterous larvæ from more than 120 cases of myiasis in man and animal, in only three cases were they those of *argyricephala*. The following notes relate to these three cases:—

*Case 1.* A number of mature larvæ of *argyricephala*, some of which had pupated, were sent by M. H. Bhatt, Veterinary Assistant, Jambusar, East Khandesh, Bombay, collected from the muscular tissue of a bullock suffering from a compound fracture.

*Case 2.* A few small third stage larvæ of *argyricephala* from a wound on a calf were sent by the same observer.

*Case 3.* A number of mature larvæ of *argyricephala* were sent by Mr. B. B. Joshi, Officer-in-charge of the Public Veterinary Hospital, Bhamburda, Poona, taken from a wound on the foot of a bullock.

In all three cases there were no larvæ of *Chrysomya bezziana* sent along with those of *Lucilia argyricephala*.

### *Lucilia argyricephala* Macq.

#### *Early Stages.*

The egg of *Lucilia argyricephala* is smaller than that of *C. bezziana* already described. When first laid, it is of a creamy white colour, but later becomes yellowish. The two chorionic ridges are somewhat closely approximated, meeting just before the blunt end of the egg, and diverging at the pointed or anterior end to enclose the micropilar opening. From 380 to 460 eggs are usually laid in a mass, and the larvæ hatch out in about 24 to 36 hours, according to the temperature.

The mature larvæ (Plate LVII, Fig. 1) measures about  $\frac{1}{2}$  in. in length and is of a dirty white colour. The head segment bears two well-developed antennæ, which consist of conical prominences, each with a minute subdorsal chitinous process. The prothorax is smooth and is not armed with spines. The anterior spiracles project from the sides of the lower border of the segment, and end in from 6 to 8 knob-like processes, more usually eight. The mesothorax, metathorax and the first eight abdominal segments are provided with about 6 to 7 rows of extremely minute, yellow, recurved spines which form belts along their anterior borders. On the ventral surfaces of abdominal segments 1 to

8 there are the usual raised pads, which are provided with the same microscopic spines. The end of the eighth abdominal segment (Plate LVII, Fig. 2) is nearly flat, sloping slightly from above downwards; it bears the two spiracular plates which are well separated and obliquely directed towards each other. The upper margin of the posterior end of this segment has three pointed, fleshy processes on each side of the middle line, slightly separated from each other.

The lower margin forms a slight shelf, and has two pointed, fleshy processes at the sides, the outermost being the more prominent. Each spiracular plate is light brown in colour, small, rounded and placed somewhat obliquely, the inner and lower angles being slightly pointed, and directed inwards; the three slits are straight and are directed obliquely towards the inner and lower angle. The ninth segment has two stout, fleshy processes on each side, and the anal slit is guarded by two raised lips.

The puparium (Plate LVII, Fig. 3) is light brown in colour, the posterior end is rounded, so that the spiracular plates are plainly visible. The processes on the eighth segment of the larva now appear as dark brown points.

#### *The Adults.*

*Male*.—Eyes reddish brown, facets of equal size. Front comparatively broad, about  $\frac{1}{4}$  width of head. Ocellar triangle dark brown, vertex bright metallic green. Parafrontals silky white, cheeks silvery with numerous black hairs. Antennæ black, grey pollinose. Palpi dark orange with dark apices. Thorax bright metallic green with bronze sheen; presutural area pollinose white with two distinct narrow, dark green admedian bands. Abdomen metallic green with white pollinose and bronze sheen; two prominent bristles at the sides of segment two, and four at sides of the lower border of the upper surface of segment three. A prominent bristle at each side of apical end of segment four. Legs black, ventral and outer surfaces of fore femora markedly metallic green, mid and hind femora less so. Wings hyaline, veins light brown near base, venation as in drawing. Squamæ dirty white.

*Female*. (Plate LVII, Fig. 4.) Front wide, about  $\frac{1}{3}$  width of head. Ocellar triangle and vertex bright metallic green. Parafrontals silvery white; cheeks yellowish white with black hairs. Bristles of abdomen less developed than in male, in all other respects like it,

*Habits of Adults.*

*Lucilia argyricephala* is the common bazaar meat and sweet fly of India; it never enters houses. The female prefers to oviposit on fresh meat, entrails and offal of all kinds usually found about Indian butchers. It will also readily oviposit in the bodies of freshly killed birds and animals, and to some extent in decomposing bodies. Mr. Bullard, F.E.S., Government Entomologist, Madras, showed me a number of typical specimens which were bred from the decomposing bodies of rabbits. I have bred it in large numbers in Madras from larvæ collected from the stomach contents of slaughtered animals, especially when soaked with blood; *argyricephala* is readily attracted to shed blood and can be easily bred by placing females in cages with some fresh meat.

Roubaud in his 'Études sur la Faune Parasitaire de l'Afrique occidentale française. Part I, Les Producteurs de Myiases et Agents similaires chez l'homme et les animaux,' states that *Lucilia argyricephala* is one of the specific myiasis-producing flies of Africa. He records a case of myiasis in a dog in which the larvæ of this species were removed from an ulcer of the lower jaw. He further mentions that Dr. Joyeau found the larvæ of *argyricephala* at Kourvoussa, French Guinea, in ulcers on a female leper, and that Dr. Brumpt commonly found the larvæ of this species in cases of myiasis in camels in Abyssinia. Roubaud also states that he has found the puparia of *argyricephala* in birds nests along with those of *Passeromyia heterochæta*, and thinks it very probable that the larvæ of *argyricephala* cause myiasis in the young birds, the larvæ developing in the wounds made by those of *Passeromyia heterochæta*. In Coonoor, I have not found either the larvæ or the puparia of *argyricephala* in the nests of sparrows in which there were many larvæ of *Passeromyia heterochæta*.

It is interesting to note, however, that Roubaud makes no mention as to whether he has found the larvæ of *argyricephala* in either fresh or decaying animal bodies. In India, it is certainly not a specific myiasis-producing calliphorine, and judging from the fact that I have only received its larvæ from three cases of myiasis, it must be considered a rare myiasis-producing species, and then only in animals. And it should be noted that the three cases are all from the Bombay Presidency. The larvæ of *argyricephala* is not well adapted to live in the tissues of man and animals, for it has very poorly developed segmental spines in comparison with those of the larvæ of *Chrysomya bezziana* in which they are highly developed.

*Lucilia argyricephala* is widely distributed in India and is found at altitudes of 6,000 feet. It is commonly infected with three distinct flagellates, *Rhynchoidomonas luciliae* Patton, in its Malpighian tubes, *Herpetomonas mirabilis* Roubaud and *Herpetomonas muscae domesticae* Burnett in its alimentary tract. I have obtained pure cultures of the two latter flagellates on the NNN medium. *H. mirabilis* grows in the culture medium with difficulty, while *H. muscae domesticae* flourishes in it. Although I have obtained a pure culture of *Rhynchoidomonas luciliae*, it never lives more than a week in the culture medium. I need hardly state again that were it only a stage in the development of *Herpetomonas muscae domesticae*, as some observers seem to think, there should be no reason why it should not develop into the *Herpetomonas* form. Further, *Herpetomonas muscae domesticae* obtained from this fly has never developed into a *Rhynchoidomonas*, though it has now been cultured for months.

*Lucilia craggii* Sp. Nov.

*Early Stages.*

The egg of *Lucilia craggii* is slightly larger than that of *argyricephala*, measuring about  $\frac{1}{12}$  in. in length: it is of a light yellow colour. The chorionic ridges are narrowly separated, uniting just before the rounded end of the egg, and separating at the pointed end to enclose the micropilar opening. The number of eggs laid by a single female varies according to its size, counts of 356 to 780 represent the extremes. They are laid in a mass, usually out of sight, on the under side of the body of a bird or animal, close to the bases of feathers or hairs, as the case may be. It is quite common to find as many as ten females ovipositing together at the same spot, and one or more may become caught in the masses of egg laid around them. The eggs are sometimes laid in the nostrils.

The mature larva (Plate LVII, Fig. 5) measures about  $\frac{2}{3}$  in. in length and is of a creamy white colour. The antennae form two large divergent conical prominences on each side of the head segment, each with a minute subapical process. There is a broad girdle of minute recurved spines at the lower border of the head segment. Prothorax with a ventral band of the same minute spines. The anterior spiracles project from the sides of the lower border of the segment, and are of a light yellow colour, consisting of 7 to 8 finger-like processes. The mesothorax, metathorax and the first eight abdominal segments

are all provided with anterior girdles of 8 to 10 rows of minute recurved spines; the girdle is not so well developed on the last three abdominal segments. All the abdominal segments are provided with a pad on their ventral surfaces. The end of the eighth abdominal segment (Plate LVII, Fig. 6) is distinctly hollowed out, and the posterior spiracular plates are situated in the hollow. The anterior border of the segment slightly overhangs the hollow, and the posterior border forms a ledge to it, so that, when both surfaces are brought together, the spiracular area is closed. The anterior border of the segment has six pointed, fleshy processes, three on each side of the middle line: they are much better developed than in the larva of *argyricephala*. The posterior border also has six processes, one small one on each side of the middle line, and two larger at the sides. The spiracular plates are situated in the hollow, much nearer the anterior than the posterior border; they are rounded above and externally, and pointed towards their inner and lower angles. They are well separated and distinctly larger than those of *argyricephala*; the slits are straight and are directed downwards and inwards. The anal segment is covered with minute spines ventrally, and has a long, divergent, fleshy process on each side of the segment.

The puparium (Plate LVII, Fig. 7) is light brown in colour and has the same processes as the larva at the posterior end.

#### *The Adults*

**Male.**—Eyes reddish brown, facets of equal size. Front very narrow, and eyes almost meeting just before the ocellar triangle, which has a blue sheen. Parafrontals silvery white, cheeks silvery to dark grey with black hairs. Antennæ dark grey almost black; arista light brown. Palpi light orange, darker on upper surface. Thorax dark blue with green reflections in some lights, and most marked in recently hatched specimens. Presutural area with white pollinose. Abdomen prussian blue with green reflections in some lights. In all old specimens the thorax and abdomen become of a purplish colour. Legs black. Wings hyaline, veins well marked and dark at bases. Squama dirty white.

**Female.**—(Plate LVII, Fig. 8). Front about  $\frac{1}{2}$ th width of head. Frontal stripe broad and of a dark grey colour. Ocellar triangle elongated and with blue reflection. Parafrontals narrow and black as in male. Antennæ black; palpi a little darker orange than in male. In all other respects like it.

*Habits of Adults.*

*Lucilia craggii* is an undescribed species and is the common blow fly of the South Indian Hill Stations, occurring on the Pulneys, Shevroys, and Nilgiris. Recently Major F. W. Cragg, I.M.S., after whom I have much pleasure in naming it, sent me some typical specimens from Kasauli, Punjab; he informs me it is common there in September and October. In Coonoor, it is present throughout the year. Mr. Senior-White kindly showed me specimens of this species which he collected in Shillong in October. It is very similar in its habits to the well-known European blow flies, *Calliphora erythrocephala* and *C. vomitoria*. The females regularly enter rooms on warm, sunny days, particularly dining-rooms, attracted by the smell of food, especially meat. They will readily oviposit on stale meat, and if the meat safe is left open, will lay their eggs on cooked or uncooked meat. It is quite common to find a mass of its eggs on meat, especially on fat, at the butchery, Coonoor. *Lucilia craggii* breeds in nature in the dead bodies of birds and small animals, and its larvæ are most efficient scavengers. I have bred it in large numbers in decomposing bodies of rabbits. As far as I am aware, it does not oviposit in living tissues. Both sexes are commonly infected with *Herpetomonas mirabilis* and *H. muscae domesticæ*.

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## SOME NOTES ON INDIAN CALLIPHORINÆ.

### PART III.

CHRYSOMYIA MEGACEPHALA FABR. (DUX ESCH), THE  
COMMON INDIAN BLUE BOTTLE, WHOSE LARVÆ  
OCCASIONALLY CAUSE CUTANEOUS MYIASIS IN  
ANIMALS AND CHRYSOMYIA NIGRICEPS SP.  
NOV., THE COMMON BLUE BOTTLE  
OF THE NILGIRIS.

BY

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ILLUSTRATED

BY

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[Received for publication, January 13, 1921.]

IN Part II of this series, I described and illustrated two species of *Lucilia*, *L. acyroccephala* Macq., and *L. craggi* Patton, and recorded three cases of cutaneous myiasis in animals from the Bombay Presidency caused by the larvæ of the former. Although *acyroccephala* is known to be a myiasis-producing calliphorine in Africa, I believe this is the first record of the occurrence of its larvæ in the tissues of animals in India. There can, however, be little doubt that when its larvæ are better known, they will be found in many more cases of animal myiasis in India and, perhaps, also in cases of human myiasis. The larvæ of *Lucilia cragga* have not been received from any case of myiasis.

In this paper I propose describing and illustrating *Chrysomya megacephala* Fabr., the common Blue Bottle fly of India, and recording three cases of myiasis in animals caused by its larvæ. The common Blue Bottle of the Nilgiri Hills, which is a new species, is also described and illustrated. The following notes relate to the two cases mentioned above.

*Case 1.*—A number of living and dead larvæ of *C. megacephala* were sent to me by Mr. B. B. Joshi, Officer-in-charge of the Public Veterinary Hospital, Bhamburda, Poona, collected from a wound on the horn of a bull calf.

*Case 2.*—Large number of the first and second stage larvæ of *megacephala* were sent by the same officer collected from wounds in the vagina of a cow which had recently calved.

*Case 3.*—A number of living larvæ, third stage larvæ of *C. megacephala*, and some puparia were sent by C. Dixon, Veterinary Assistant, Ahmedabad. The larvæ were collected from an unhealthy ulcer on the back of a donkey.

#### *Chrysomya Megacephala Fabr.*

##### *Early Stages.*

The egg of *Chrysomya megacephala* closely simulates that of *C. bezziana* already described and figured in Part I of this series; it measures 1-16th in. in length and is of a lemon yellow colour. The two chorionic ridges are well marked, and if anything closer together than in the egg of *bezziana*. The eggs are laid in a mass, and the number varies from 400 to 650. The larvæ hatch out in about 24 hours, according to the temperature.

The mature larva (Plate LVIII, Fig. 1) measures 9-16th in. in length and is of a white colour. The antennæ project as two well-developed prominences on each side of the oral opening. The prothorax is armed with a narrow belt of many rows of minute, brown, recurved spines. The anterior spiracles, which project from the sides of the lower border of the segment, are distinctly yellowish and consist of 12 knob-like processes, sometimes 11, and often 13. The mesothorax, metathorax and first seven abdominal segments have on each side in front of the girdle of spines a small pad which is also armed with rows of spines. On their ventral surfaces there is also the usual pad covered with spines. The eighth segment (Plate LVIII, Fig. 2) is only armed with spines on its

Fig. 4. *Chrysomya megacephala* ♀ × 6.

Fig. 9. Mature larva of *C. megacephala* × 8.

Fig. 37. Puparium of *C. megacephala* × 8.

Fig. 7. Puparium of *C. nigriceps* × 6.

Fig. 8. Mature larva of *C. nigriceps* × 8.

Fig. 8. *Chrysomya nigriceps* ♀ × 7.

Fig. 2. End of larva of *C. megacephala* × 10.

Fig. 6. End of larva of *C. nigriceps* × 10.

Fig. 4. *Chrysomys megarhina* ♀.

Fig. 5. *Chrysomys megarhina* ♂.

Fig. 6. *Chrysomys megarhina* ♂.

Fig. 7. *Chrysomys megarhina* ♂.

Fig. 8. *Chrysomys megarhina* ♂.

Fig. 9. *Chrysomys megarhina* ♂.

Fig. 10. *Chrysomys megarhina* ♂.





lateral and ventral surfaces. The end of the segment is deeply concave, the anterior lip has three pointed, fleshy processes on each side, the central being the smallest. The lower lip also has three pointed processes, a small one on each side of the middle line and two larger ones at the sides. The spiracular plates, which line in the hollow, are exceptionally large, and placed close together. Each plate is somewhat D-shaped and is surrounded by a black rim of chitin, which is wanting at the lower and inner angle. The three slits are broad and mahogany brown in colour. In the drawing the plates are shown a little closer together than they usually are. The ninth segment forms a well-marked projection below the eighth, and has a long pointed process on each side; its lower and anterior surface is covered with dark, recurved spines.

The puparium (Plate LVIII, Fig. 3) is mahogany brown in colour, and has the same markings and processes as the larvæ. The anterior spiracles project from the anterior extremity and are distinct yellow, fan-shaped processes.

#### *The Adults.*

*Male*.—Eyes in life of a characteristic brick red and closely approximated, with a band of large lenses occupying about  $\frac{3}{4}$  length of eye, but not extending to the outer or occipital margin, remaining lenses very small and completely surrounding the large lens area which is of a brighter colour than the small of small lenses. Ocellar triangle prominent, ocelli amber coloured. Parafrontals silvery. Third segment of antenna usually light orange, but in many specimens the upper surface is dark; antsa dark. Cheeks creamy buff with long yellow hairs; palpi orange. Thorax dark green with blue and bronze reflections; presutural area dusted with white pollinose, with a pair of dark admedian bands and a dark triangular patch behind the humerus on each side. Scutellum steel blue with green reflections. Abdomen dark green with blue reflections and with dark blue basal horizontal bands to segments two and three; last segment with white pollinose and pinkish reflections in fresh specimens. Legs dark brown; femora with some green reflections. Wings hyaline; veins dark at their bases. Squamæ ochraceous with dark hairs.

*Female*.—(Plate LVIII, Fig. 4). Front wide about  $\frac{1}{2}$  width of head, frontal stripe reddish brown to dark brown, widest about the middle. Parafrontals creamy yellow; cheeks buff with yellow hairs; in all other respects like the male.

*Habits of Adults.*

*Chrysomyia megacephala* is the common bazaar Blue Bottle and may be seen in large numbers sitting on meat and sweets. Fletcher, referring to it under the name *fiaviceps* in 'Some South Indian Insects,' says, 'It has proved a serious pest to toddy in South Kanara, sucking all the juice exuding from the palm spathes, fouling the pots, spathes and juice with excrementitious matter and swarming around the vessels.'

I have caught large numbers of both sexes on the decomposing bodies of animals on which the females readily oviposit. Mr. Ballard has found this species breeding in the manure heap at the Agricultural College, Coimbatore. I have had large numbers of the larvæ sent me by the Veterinary Assistant, Ongole, collected from the bodies of dead cattle, and Mr. Joshi, Poona, has sent me the adult flies caught on decomposing bodies of cattle at Poona. *C. megacephala* is therefore primarily a necrophagous fly, and breeds in a variety of food stuffs, but mainly in decomposing animal matter. It appears only occasionally to lay its eggs on diseased tissues of animals. It is found throughout India, Burma, Assam and Ceylon. Mr. Froggatt recently sent me a typical specimen from the Hebrides. It is recorded from Guinea and Java.

Both sexes are commonly infected with *Herpetomonas mirabilis* Roubaud and *Herpetomonas muscae domesticae*.

*Chrysomyia nigriceps*, Sy. Nov.*Early Stages.*

The egg of *nigriceps* is of a lemon yellow colour, and measures 1-16th in. in length. The chorionic ridges are closely approximated, uniting just before the blunt end, and diverging at the anterior end to enclose the micropilar opening. The counts of eggs laid by a number of females varied from 760 to 880. The eggs are always laid out of sight under the body of a bird or animal. As many as thirty females have been observed laying their eggs together in one mass, and several of these became imprisoned in the masses of eggs laid around them.

The mature larva (Plate LVIII, Fig. 5) measures 7-12ths in. in length and is of a creamy yellow colour. The prothorax has a dorsal belt of minute, dark brown, recurved spines, but is smooth on the ventral side. The anterior spiracles project from the sides of the lower border, and each

consists of a fan-shaped yellow process with 12 finger-like projections. The mesothorax, metathorax and the first seven abdominal segments each have a belt of small, dark, brown recurved spines in six or seven rows; the spines are, however, not so well developed as in the larva of *C. megacephala*; they are wanting on the dorsal surface of the sixth and seventh segments. There is a small pad with several rows of spines just in front of the belts on each of the first six abdominal segments. Each segment also has a well developed ventral pad armed with several rows of spines. The end of the eighth segment (Plate LVIII, Fig. 6) forms a shallow depression. The anterior lip has three pointed processes on each side of the middle line, but not so well developed as in the case of the larva of *C. megacephala*; the middle process is small and usually more or less blunt. The ventral lip has a small pointed process just external to the middle line on each side, and two longer ones at the sides.

The spiracular plates are large but not so large as those of the larva of *megacephala* and are a little further apart. Each plate is pear-shaped and has a well marked border of dark chitin, interrupted at the lower and inner angle. The slits are broad and dark brown in colour. The ninth segment is prominent, and has a pointed, fleshy process on each side. It is armed with dark recurved spines on its lower and anterior surfaces.

The puparium (Plate LVIII, Fig. 7) is mahogany brown in colour, and has the same markings and processes as the larva.

#### *Adults.*

*Male*.—Eyes closely approximated and reddish brown in life with an area of large lenses extending about  $\frac{1}{3}$  length of the eye, but not reaching the sides and occipital margin, the remaining lenses are small and completely surrounding the large lens area. Ocellar triangle dark, ocelli amber coloured. Parafrontals dark orange, cheeks dark grey to black with some yellow dusting and covered with golden hairs. Antenna dark orange; upper surfaces dark grey to black; arista dark grey; palpi dark orange. Thorax dark blue with some green reflections; presutural area with white dusting anteriorly and two narrow dark blue admedian bands, and a triangular dark blue patch behind each humerus; scutellum dark blue. Abdomen dark blue with some green reflection and dark bands to lower borders of segments two and three. In freshly hatched specimens the last segment is whitish with some pink reflection.

Legs black, femora with some blue reflections. Wings hyaline, veins well marked, basal points light brown; squamæ ochraceous with dark hairs.

*Female.* (Plate LVIII, Fig. 8.) Front wide about 1-3rd width of head, dark, grey to black with some bluish reflections at vertex. Frontal stripe wide greyish black; parafrontals dark grey. Cheeks greyish brown with yellow hairs. In all other respects similar to the male.

*Habits of Adults.*

Dr. Villeneuve informs me that this *Chrysomyia* is an undescribed species, and I propose naming it *nigriceps*. The females only occasionally enter houses attracted by the smell of food. It breeds in nature in the dead bodies of birds and small animals, and its larvæ are very efficient scavengers. I have bred it in large numbers in the bodies of rabbits. I have taken specimens at Kallar at the foot of the Coonoor Ghat about 2,000 feet. Mr. Senior-Whit collected a number of specimens in Shillong in October and I have seen specimens from Darjeeling.

# SOME NOTES ON INDIAN CALLIPHORINÆ.

## PART IV.

### CHRYSOMYIA ALBICEPS WIED. (RUFIFACIES FROGGATT); ONE OF THE AUSTRALIAN SHEEP MAGGOT FLIES AND CHRYSOMYIA VILLENEUVII. SP. NOV.

BY

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ILLUSTRATED

BY

EDITH M. PATTON.

[Received for publication, January 13, 1921.]

IN Part III of this series I described *Chrysomya megeri-plala* Esch., and recorded for the first time three cases of cutaneous myiasis in animals caused by its larvæ. It will be remembered that this species has been in the past confused with *C. flacciceps*, which is distinct, and, as far as I have been able to ascertain does not occur in India. I also described a new species of blow fly, *C. nigriceps*, from Coonoor.

In the present paper I propose describing and illustrating *Chrysomya albiceps* Wied., a widely distributed blow fly, and one of the well-known Sheep Maggot Flies of Australia where it is known by the name of *rufifacies* Froggatt; and another new species from South India, which I have much pleasure in naming after my friend Dr. Villeneuve.

Both these species are of peculiar interest, for they have very characteristic predaceous third stage larvæ, which are quite unlike any of the larvæ of the other species of *Chrysomya* yet described.

*Chrysomya albiceps* Wied.

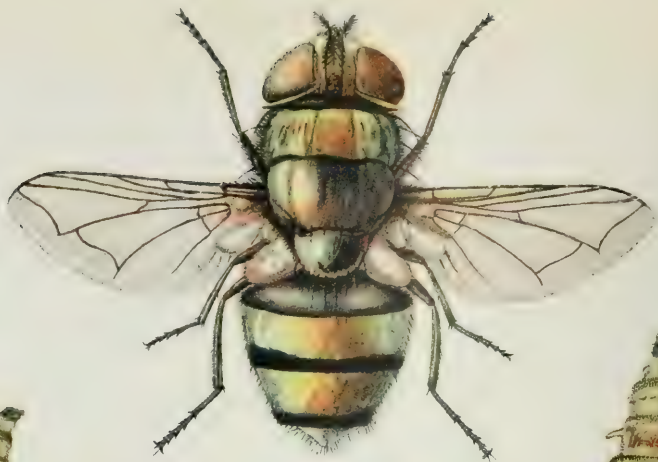
*Early Stages.*

The egg of *albiceps* measures  $\frac{1}{16}$  in. in length and is slightly broader than the eggs of the other species of Calliphorinæ: it is of a lemon yellow colour. The chorionic ridges are closely approximated, meeting just before the posterior end of the egg, and diverging slightly at the anterior end to enclose the micropilar opening. The eggs hatch in from 24 to 36 hours, according to the temperature. They are laid, as a rule, among the eggs of other species, but may be laid separately in a mass. I have not been able to count the exact number of eggs laid by a female, but from dissections it would appear that they may vary from 300 to 450.

The first stage larva is structurally like that of *C. megacephala* and *C. nigriceps*, and has none of the fleshy processes seen in the third stage. These only develop in the second instar in which they are small, only reaching their full size in the third stage.

The mature larva of *albiceps* (Plate LIX, Fig. 1) measures a little less than  $\frac{1}{2}$  in. in length, and is of a greyish yellow colour, the dorsal surface is marked with dark patches, and the ventral creamy white. The anterior end is markedly attenuated. The antennæ consist of two blunt prominences, each with a small subapical blunt process. There is a broad belt of black, recurved spines behind the head segment, they are smaller on the lateral and ventral surfaces but larger on the dorsal face. At the anterior end of the prothorax there is, on each side, a row of very small, blunt, fleshy processes, two dorso-lateral and two ventro-lateral. The yellow, fan-shaped, anterior spiracles project from the sides of the lower border of the segment, and consist of from 9 to 11 small, button-like processes. The mesothorax has a well marked belt of from 8 to 10 rows of black, recurved spines at its anterior margin: the spines are larger and more numerous on the dorsal than the ventral surface. Just behind the belt, there is a row of eight blunt, fleshy processes, four on each side, two dorso-lateral and two ventro-lateral; the upper ventro-lateral process is the more prominent of the two. The metathorax is similarly armed with an





125

Fig. 3. *Chrysomys albiceps* × 7.

Fig. 1. Mature  
larva of  
*C. albiceps* × 6.

Fig. 2. Pupa of  
larva of  
*C. albiceps* × 10.

Fig. 3. Pupa of  
larva of  
*C. villosus* × 10.

Fig. 4. Mature  
larva of  
*C. villosus* × 6.

Fig. 5. *Chrysomys villosus* × 7.

Fig. 3. *Chrysomya albiceps*  $\times 7$ .

Fig. 1. Mature  
larva of  
*C. albiceps*  $\times 6$ .

Fig. 2. End of  
larva of  
*albiceps*  $\times 10$ .

Fig. 5. End of  
larva of  
*villeneuvei*  $\times 10$ .

Fig. 4. Mature  
larva of  
*C. villeneuvei*  $\times 6$ .

Fig. 6. *Chrysomya villeneuvei*  $\times 7$ .

anterior belt of recurved spines, which are poorly developed on the ventral surface. In addition, the whole dorsal surface of the segment is covered with small black spines and there is a dark yellow band occupying the middle of the dorsal surface. Behind the belt of spines there is a row of eight more elongated, fleshy processes, four on each side, two dorso-lateral and two ventro-lateral. Each process is smooth and has a tuft of black spines at its apex. The upper ventro-lateral process is the more elongated.

The first abdominal segment has a narrow belt of spines which is better marked on the ventral surface, and the whole of the dorsal and lateral surfaces of the segment are covered with small dark spines. There is a row of eight long, fleshy processes projecting backwards as in the metathorax, each with a smooth stalk and a tuft of black spines at its apex. The fourth process, which is situated ventro-laterally, has a smaller process, arising from its base just behind the anterior border of the segment. Situated on a ridge on the ventral surface of the segment there is a row of six small blunt processes, three on each side of the middle line. The abdominal segments 2 to 7 inclusive, are similarly armed, and there is the usual pad covered with spines on the ventral surfaces of the segments. The dorsal surfaces of the segments are dark, the colouring forming more or less distinct spots on each side of the middle line as is shewn in the drawing. The end of the eighth segment (Plate LIX, Fig. 2) is deeply hollowed out. The anterior lip has three long, fleshy processes on each side, each with a tuft of spines at its apex. The lower lip has two long, lateral processes, the outer the longer of the two: below the lower lip and above the ninth segment there is a process on each side of the middle line. The hollow is blotched and streaked with yellowish brown patches of pigment.

The posterior spiracles are somewhat small for the size of the larva, each is pear-shaped and has a continuous, broad rim of dark elatrin; the three slits are broad and dark brown in colour. The ninth segment is very prominent, and is covered with black, recurved spines, and has a small, fleshy process on each side.

The second, but more particularly the third, stage larva of *albiceps* is entirely predaceous, feeding on the larvae of other *Calliphoridae*. I first observed it in Mesopotamia, where it was common to find it feeding on the larvae of *Musca determinata* and *M. hordae* in heaps of horse manure. In Coonoor, I have been able to study its habits more

carefully by laying out a number of decomposing bodies of rabbits. The female fly, on coming to the bait, hunts about until she finds a number of other Calliphorinæ laying their eggs, she then lays hers among theirs, and I have observed the eggs being laid among those of *C. megacephala*, *C. nigriceps* and *L. cragii*. The first stage larva feeds on the decomposing body, and after about 36 hours, changes to the second stage; it then begins feeding on the larvæ of the other species. This stage lasts about 2 to 3 days and the third stage larva now feeds entirely on the other larvæ. When a larva comes within its reach it curls round it, grasping it firmly with the spiny ninth segment, and immediately burying its mouth parts into the larva, nearly always in the metathorax or first abdominal segment; it holds it in this way until it has sucked out all its juices. I have hardly ever seen a larva escape once it has been caught. It will readily attack a larva twice its size, such as a mature larva of *Sarcophaga*. Structurally, it is well adapted for this habit, for it will be noted that the head end is markedly attenuated for penetration into the tissues of its prey. I will, on another occasion, describe the mouth parts and sucking apparatus of this larva and those of the next.

The puparium is dark brown in colour, the dorsal surface markedly convex; it has the same processes as described for the larva and can always be recognized by noting that they are smooth except for the apex which has a group of dark spines.

#### *The Adults.*

*Male*.—Eyes reddish brown; approximated, lenses of equal size. Vertex dark green, sometimes bluish; ocellar triangle black; ocelli amber coloured. Parafrontals light to dark grey with silvery hairs. Cheek white with long silvery hairs. Antennæ dark orange with basal segments reddish brown; arista dark grey. Palpi light orange. Thorax green, often dark green with blue bronze reflections; presutural area with white dusting and two narrow, dark green, admedian bands, and a triangular dark patch behind each humerus; thoracic stigmata yellowish white. Abdomen green with bronze reflections, and broad, dark blue bands at the lower borders of segments two and three. Last segment with white dusting and pink reflections in fresh specimens. Legs black, all femora with green reflections. Wings hyaline, fourth vein with very short appendix at bend, more marked in the male, and usually present in all specimens though it may be very weakly developed.

*Female*.—(Plate LIX, Fig. 3.) Front wide about  $\frac{1}{2}$  width of head ; vertex metallic green with bluish reflection. Frontal stripe narrow, dark grey to black ; parafrontals silvery grey to dark grey ; cheeks silvery and near middle line with a dark orange tinge when seen from below. Bands on thorax and abdominal segments well marked, especially those of the latter ; in all other respects like the male.

#### *Habits of the Adults.*

*Chrysomya albiceps* is one of the important necrophagous calliphorines of India. Its larvæ are commonly found in company with those of *megacephale* and in Coonoor with *C. nigriceps*. It does not appear to breed in recently dead animals, but only when decomposition is well advanced. I have only seen a few specimens from meat stalls in the bazars, and have never obtained its larvæ from offal or bits of meat which are commonly found about Indian butcheries. *C. albiceps* is widely distributed in India.

Recently I have had the opportunity of examining a collection of Australian Calliphorinæ kindly sent me by Mr. W. W. Froggatt, whose work on the Australian species is so well known. On comparing *albiceps* with *rufifacies* Guerin, it was at once clear that they are identical. Mr. Froggatt also sent me the third stage larva of *rufifacies*, which he has described, and it is exactly similar in every detail with the third stage larva of *albiceps* which I have reared in India and in Mesopotamia. *Chrysomya rufifacies* must therefore in future be known as *albiceps*.

In Australia, this species is, according to Mr. Froggatt, one of the important sheep-maggot flies. Mr. Froggatt states that in 1905, it was not one of those known as an active agent in the work of blowing soiled wool on living sheep. It was not among those recognised as sheep flies in the paper, published in the Gazette for January 1905, and as late as 1910 when I furnished another contribution, "Sheep Maggot Fly in the West," we had not bred this species from maggots in soiled wool, though in the West it was very plentiful about killing yards, freshly skinned sheep and dead animals.

The publication of the notes in 1910 brought much interesting information and material from sheep owners, and we soon found from the infested wool thus received that the so-called "barry maggots" well known to the squatters, were the larvae of this fly and that it also bred in soiled wool. But at that date they were not as common as the two other

species of yellow house blow flies, *Calliphora oceanica* (*Anastellorrhina augur*), *C. villosa* (*Pollenia stygia*), which up to this date had been the chief culprits.

This is another case of the adaptation of new habits by a previously harmless insect, on account of new conditions arising—in this case the presence of smelly wool. From our present investigations in the northern and western districts of this State, and western Queensland, it is evident that this is now the common sheep-maggot fly, while the two other species, from which it probably first acquired the habit, have almost entirely disappeared from this great area during the early summer months. At the time of writing, they certainly do very little damage for six months in the year in comparison with *C. rufifacies*.<sup>3</sup>

It will be noted from the above remarks that *albiceps* is considered a serious pest in Australia, and that, according to Mr. Froggatt, it has only within recent years taken to laying its eggs in soiled wool. Knowing the habits of its third stage larvæ in India and Mesopotamia, it seems curious that there is no record of their feeding on those of other Calliphorinæ in Australia. Mr. Froggatt believes the female fly has acquired the habit of laying its eggs in soiled wool from the two other flies mentioned above.

It seems to me, however, that another explanation may be given for this newly acquired habit. The female *albiceps* may have laid its eggs among the eggs and larvæ of other Calliphorinæ in soiled wool, so that its third stage larvæ may obtain as their normal food the larvæ of other Calliphorinæ. Having once discovered this plentiful supply of food for its second and third stage larvæ, the fly would rapidly multiply, reducing the other species in numbers, which Mr. Froggatt's statements suggest. It would be interesting to know whether the larvæ of *albiceps* are commonly found in soiled wool in the absence of the larvæ of any other species. If this is the case, and Mr. Froggatt seems to suggest this, then the third stage larva of *albiceps* in Australia has become necrophagous in habit. But it appears possible that, when in large numbers, it would be difficult to find any other larvæ with it, as it would have destroyed them; this is an interesting problem and would, I believe, well repay further investigation. In any case, the fact that *rufifacies* is identical with *albiceps* is of considerable importance to India, for this fly may, in this country, acquire similar habits.

*Chrysomyia villeneuvei* Sp. Nov.*Early Stages.*

The egg of *C. villeneuvei* measures  $\frac{1}{2}$  in. in length and is very similar in structure to that of other species already described. The two chorionic ridges are, if anything, more closely approximated.

The mature larva of *villeneuvei* (Plate LIX, Fig. 4) measures  $\frac{1}{2}$  in. in length and is of a dark grey colour throughout, the middle of the segments being, if anything, darker; the anterior end is markedly attenuated. The antennae consist of two prominent processes on each side of the head segment, posterior to which there is a well-developed girdle of strong, black, recurved spines, which are better marked on the dorsal than on the ventral surface. The prothorax has a row of short, blunt processes at its anterior end, two dorso-lateral and two ventro-lateral. The fan-shaped, yellowish, anterior, spiracular processes project from each side of the lower border of the segments, each ending in 14 small stigmatic processes. The mesothorax has an anterior girdle of dark, recurved spines, and behind it a row of better developed, blunt processes as in the prothorax. The metathorax is without a girdle of spines, the whole segment being covered with minute, dark, straight denticles; it also has a row of fleshy processes, the dorsal one is more elongated and covered with minute, black denticles with a distinct apical tuft of larger ones; the dorso-lateral process is longer than the lower one. On the ventral surface of the segment situated on a ridge there is a faint identification of two knob-like processes on each side of the middle line.

The first abdominal segment is similarly armed with two dorsal and two lateral longer backwardly-directed processes, the dorsal being much longer than the lateral. The lower lateral process has a smaller process arising from its base. On the ventral surface there is a row of six blunt processes on a ridge, three on each side. The abdominal segments, 2 to 7, inclusive, are similarly armed. The eighth segment has no tubercles on its ventral surface, but only a ridge with well-developed spines. The end of the segment (Plate LIX, Fig. 5) is deeply hollowed out, the dorsal lip overhanging it. It is armed with three long fleshy processes on each side, the third being situated at the edge. The lower lip forms a long, broad ledge, and has two long processes at the sides, and two below the edge on each side of the middle line. The posterior spiracles are situated in the hollow, and consist of two almost round, broad, chitinous rings of a

dark brown or reddish brown colour. The three slits are broad and of a lighter colour. The ninth segment is prominent and is covered with strong black denticles, and has two small, fleshy processes at the sides. The segment is used by the larva in progression, and more particularly in holding its prey.

The first stage larva of *villeneuvei* is exactly similar in general structure to that of the other species described in these notes; it is a short stage, lasting about 36 hours. The second stage has small, fleshy processes like the mature larva, but these only reach their full development in the last instar. The second instar, but more particularly the third, is extremely perlaceous. A few of these will, in a very short time, destroy a large number of other larvæ in a decomposing body; and they will readily feed on the larvæ of *albiceps* in the absence of others. Text Fig. 1 shows one of these larvæ feeding on large *Sarcophaga* larva; its method of holding its prey with the spiny ninth segment is well shown in the drawing. It is not uncommon to find two larvæ of *villeneuvei* feeding on the same larva (Text Fig. 2).



Text Fig. 1. Mature larva of *C. villeneuvei* feeding on a larva of *Sarcophaga*.



Text Fig. 2. Two larvæ of *C. villeneuvei* feeding on a larva of *Sarcophaga*.

The third stage larvæ of *C. villeneuvei*, like those of *C. albiceps*, are certainly useful in India, as they destroy a large number of other blow fly larvæ.

The puparium is of a dark colour, and never becomes mahogany brown. It has all the spines and processes of the mature larva, so that these need not be described again.

#### *The Adults.*

*Male.*—Eyes widely separated, reddish brown, and lenses of equal size. Front a little more than  $\frac{1}{2}$  width of head, greyish white to

yellowish white. Vertex dark with metallic sheen on each side of ocellar triangle. Frontal stripe dark brown; parafrontals greyish yellow. Cheeks blackish with long yellow hairs. Antennae dark grey to black; basal segments and upper portion of third segment dark orange. Epistoma dark orange. Palpi dark orange with black bristles. Thorax dark metallic blue with green reflections. Presutural area with two narrow dark green admedian bands, and a dark green triangular patch behind each humerus. Abdomen dark metallic blue with green and violet reflections; narrow dark bands at bases of segments two and three. Whole abdomen covered with long black hairs which form a characteristic fringe all round it. Legs black, extremely well developed; all femora metallic blue, markedly dilated, and armed with a fringe of long black hairs on upper and lower borders; tarsi well developed and claws unusually large. Wings hyaline, all veins strongly developed; squamæ yellowish white with light hairs.

*Female*.—(Plate LIX, Fig. 6). Front wide a little more than  $\frac{1}{3}$  width of head. Metallic reflections on sides of ocellar triangle well marked; ocelli large and bright amber coloured. Parafrontals creamy; cheeks dark with yellow hairs. Abdomen broader than in male, in which it is characteristically rounded at sides; fringe of hairs wanting, thus affording a simple means of separating the sexes, as the front of the male is broad.

#### *Habits of Adults.*

This species was first seen in Coonoor in March, both sexes being attracted to rabbits' bodies in an advanced stage of decomposition. Since then I have taken number of specimens at Kallar (2,000 feet) at the foot of the Coonoor Ghat. I have not seen any specimens from any other locality, and it does not frequent bazaars. I have much pleasure in naming it in honour of Dr. Villeneuve who examined it for me and confirmed my opinion that it was new to Science. It is a very striking fly, its stout metallic femora, well-developed tarsi and rich colour rendering identification easy.

By laying out numerous baits of decomposing bodies of rabbits, I have been able to study its breeding habits very thoroughly. The female fly lays its eggs in small batches among the eggs of other calliphorine, and the second stage, and particularly the third, are extremely predaceous, only feeding on the larvæ of other blow flies and those of *Sarcophaga*.

It would be interesting to know whether it is found in other parts of India.

# SOME NOTES ON INDIAN CALLIPHORINÆ.

## PART V.

LUCILIA PULCHRA WIED. (RUFICORNIS MACQ.). A  
LARVIPAROUS CALLIPHORINE. AND LUCILIA  
BALLARDII, SP. NOV., A COMMON SOUTH  
INDIAN BLOW FLY.

BY

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ILLUSTRATED

BY

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[Received for publication, January 13, 1921.]

IN Part IV of these notes, I described *Chrysomyia albiceps* Wied. (*ruffacies* Guérin), and a new species *C. villeneuvei* Patton, the third stage larvæ of which are structurally very characteristic, being armed with long fleshy processes, and are extremely predaceous, feeding on other necrophagous and coprophagous larvæ. *C. albiceps*, it will be remembered, is one of the sheep-maggot flies of Australia, only having acquired the habit of laying its eggs in soiled wool within recent years. There is no record, however, as to whether its third stage larva is predaceous in Australia, but the fact that where it exists other sheep-maggot flies, notably *Anastolorrhina augur* and *Pollenia stygia*, seem to be greatly reduced in numbers, suggests that its larva may have the same habit in Australia as it has in India and Mesopotamia.





FIG 4



FIG 1.



FIG 3



FIG 7.



FIG 5.

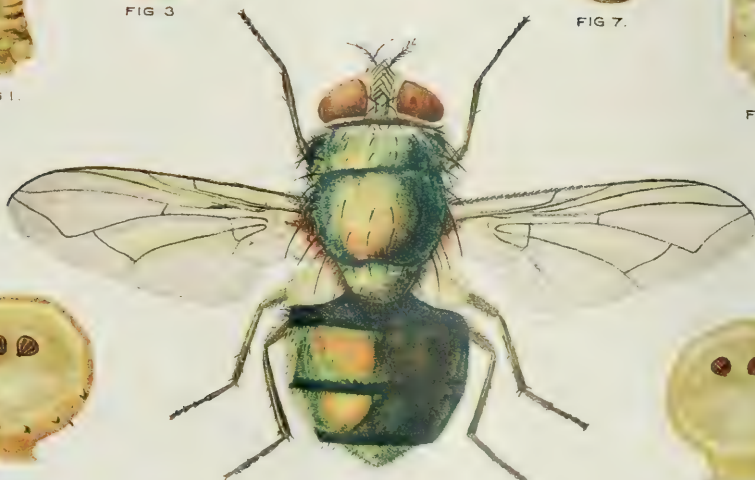


FIG 8



FIG 2



FIG 6.

Fig. 1. *Lucilia pallardi* ♂ x 7

Fig. 1. Mature  
larva of  
*L. pallardi* ♂

Fig. 2. Puparium  
of *L. pallardi* ♂

Fig. 3. Puparium  
of *L. pallardi* ♂

Fig. 4. Mature  
larva of  
*L. pallardi* ♂

Fig. 5. *Lucilia pallardi* ♀ x 8

Fig. 6. Mature  
larva of *L. pallardi* ♂

Fig. 6. Head of larva of  
*L. pallardi* ♂ about 12

4. *Lucilia pulchra* ♀ × 7

Fig. 1. Mature  
larva of  
*L. pulchra* × 6.

Fig. 3. Puparium  
of *L. pulchra* × 5.

Fig. 7. Puparium  
of *L. ballardii* × 5.

Fig. 5. Mature  
larva of  
*L. ballardii* × 5.

Fig. 8. *Lucilia ballardii* ♀ × 6.

Fig. 2. End of larva of  
*L. pulchra* × about 12.

Fig. 6. End of larva of  
*L. ballardii* × about 12.

In the present paper I propose describing one of the handsomest Indian blow flies, *Lucilia pulchra* Wied., and another new species of *Lucilia* from South India.

*Lucilia Pulchra* Wied.

*Early Stages.*

As this species only occurs in the plains, I am indebted to Mr. Ballard, F.E.S., Government Entomologist, Madras, for specimens of its first and third stage larvæ, many adults and notes on its breeding habits. This species is viviparous and deposits its first stage larvæ one at a time, either on human excrement, or decomposing bodies of birds and animals.

The mature larva (Plate LX, Fig. 1) measures  $\frac{1}{2}$  in. in length, and is of a dirty yellow colour. The antennæ consist of two large prominent knob-like processes. The prothorax has a narrow belt of very minute spines at its anterior end. The anterior spiracles project from the sides towards its lower end, and consist of six, sometimes seven, processes. The mesothorax is smooth, and is not armed with a belt of spines. The metathorax has a well-developed belt on its dorsal surface, and the first abdominal segment is similarly armed, but the girdle is less developed in the remaining abdominal segments. There is a well-marked pad covered with minute spines at the anterior ends of all the abdominal segments.

The end of the eighth abdominal segment (Plate LX, Fig. 2) slopes gradually from before backwards. The anterior margin has three small, pointed, fleshy processes on each side of the middle line, and the posterior margin one on each side of the middle line and two at the sides. The ninth segment is moderately well developed, and has a fleshy process on each side; it is covered with minute yellow spines. The posterior spiracular openings consist of two somewhat pear-shaped light brown plates with three straight slits directed backwards and inwards. The plates are nearer each other than those of either *L. argyricephalæ* and *L. craggii*.

The puparium (Plate LX, Fig. 3) is light brown in colour and has the same markings and processes as the mature larva.

*Adults.*

*Male.* Eyes reddish brown and closely approximated. Frontal stripe very narrow: vertex dark with some green reflections:

parafrontals silvery white. Cheeks brilliantly white with dark hairs. Antennæ bright orange; arista light with dark hairs; palpi orange. Thorax bronze in centre and greenish blue at the sides with white dusting most marked anteriorly and at the sides. Scutellum dark blue with brassy high lights. Abdomen dark green with a bright brassy sheen. Legs black; wings hyaline, veins dark brown; squamæ white with light hairs.

*Female.*—(Plate LX, Fig. 4.) Eyes reddish brown. Front wide, more than  $\frac{1}{4}$  width of head; vertex greyish with some green reflections. Parafrontals and cheeks brilliantly white and antennæ bright orange; these characters render identification very easy. Thorax light sea green, with white dusting and shimmering white sides. Upper half of abdomen dark blue, lower half green, and last segment with white dusting; otherwise it is like the male.

#### *Habits of the Adults.*

This species appears to be essentially a flower and fruit juice feeder. The females are commonly found at Coimbatore in July feeding on the fruit of the Neem tree. Its distribution in India is not known.

#### *Lucilia ballardii* Sp. Nov.

#### *Early Stages.*

The egg of this species is exactly similar to that of *L. craggi* already described. The females are attracted to human excrement and large numbers of them were caught feeding on it, at Burliar, and Kallar at the foot of the Coonoor Ghat. The eggs are, however, never laid in excrement, but only in decomposing animal matter.

The mature larva (Plate LX, Fig. 5) measures about  $\frac{1}{2}$ " in length and is of a dirty white colour. The antennæ consist of two prominent, fleshy processes. The prothorax is smooth, the anterior spiracles project from the sides and end in six processes. The meso, metathorax and first seven abdominal segments are provided with a narrow belt of small spines at their anterior borders; and is the usual fleshy pad on the ventral surfaces of the segments. The eighth abdominal segment (Plate LX, Fig. 7) slopes gently from above downwards; the anterior border has three minute, pointed, fleshy processes, the lower border one on each side of the middle line, and two at each side. The posterior

spiracles are small and well separated, a little more so than those of *Lucilia coarctata*, the chitinous rim completely surrounding the three slits. The ninth segment is not very prominent, and has two short spines at the sides.

The puparium, like that of the other species described in these notes, is of a dark brown colour, and shows the same markings and processes as the mature larva.

#### Adults.

*Male*.—Eyes reddish brown and somewhat closely approximated, facets of equal size. Front silvery white, and frontal stripe black; parafrontals and cheeks silvery, the latter covered with black hairs. Antennæ mouse grey, and arista dark brown; palpi dark orange. Thorax bright green with bronze and blue reflections, white dusting anteriorly and two faint, dark, admedian bands; scutellum dark green. Abdomen bright green with brassy reflections, and narrow, dark, horizontal bands. Legs black. Wings hyaline; squamæ dirty white.

*Female*.—(Plate LX, Fig. 8.) Front about  $\frac{1}{2}$  width of head; vertex with green reflections; frontal stripe and cheeks white, the latter with black hairs. Antennæ dark grey; palpi orange. Thorax bright green with bronze reflections, white dusting anteriorly and two narrow indistinct admedian longitudinal bands. Abdomen green with brassy reflections, some with white dusting and well marked, narrow, dark horizontal bands; first apparent segment black, otherwise like the male.

It will be noted from this description that this species of *Lucilia*, unlike most others, has characteristic dark bands on the abdominal segments and also faint thoracic stripes.

#### Habits of Adults.

This species, which I have much pleasure in naming after Mr. Ballard, is widely distributed in South India, and is very commonly seen feeding on human excrement. The eggs, however, appear to be laid only on decomposing animal matter.

Before concluding these descriptive notes, I would like to take this opportunity of thanking Mr. Ballard, F.R.S., Government Entomologist, Madras, for the trouble he has taken in breeding some of the species described at Coimbatore. Also Dr. Villeneuve for identifying the myiasis-producing species. I wish also to thank Mr. Samuel White, F.R.S.

for the trouble he has taken in looking up for me the descriptions of some of the older species. I would also like to point out that it is more than likely that some of the new species may prove to have been described long ago either by Bigot, Wiedemann or Walker, but as it is quite impossible at present to locate their species, I have considered it best to give names to those which cannot otherwise be named. The species described in these notes by no means represent all the Indian Calliphorinæ, nor even those which I have in my collection; at present it would be useless attempting to describe any more. The types of my new species are in my collection.

# ENTOMOLOGICAL NOTES ON FIELD SERVICE IN WAZIRISTAN.

BY

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[Received for publication, April 24, 1921.]

WHILE on field service in the Waziristan campaigns of 1917 and 1919-1920, I had the opportunity of collecting a number of 'sandflies' and mosquitoes from this area of the North-West Frontier Province.

As this part of India is notorious for the amount of malaria which occurs there, and as Pappataci Fever and 'Oriental Sore' are also prevalent diseases, the following notes on the occurrence of some species of phlebotomi and anophelines may be of use to other workers in those regions.

## PHLEBOTOMI.

During the summer and autumn of 1919 and the spring of 1920 I collected 736 specimens of this genus from the Waziristan area.

### (1) *Species of Phlebotomus.*

The specimens included *P. papatasi*, *P. minutus*, *P. minutus* var. *antennatus* and *P. sergenti*.

The number of specimens of each species is shown in the following table:—

SEX.	<i>P. papatasi</i> .		<i>P. minutus</i> .		<i>P. minutus</i> var. <i>antennatus</i> .		<i>P. sergenti</i> .		Total.	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Number	102	186	255	149	19	18	6	1	382	354
TOTAL	288		404		37		7		736	
TOTAL PER CENT	39.13		54.90		5.02		0.95		100	

From an examination of the specimens of *P. antennatus*, Newstead, and a comparison of them with *P. minutus* it was decided that difference in length of the antennal segments by which these two insects were differentiated could not be maintained as of specific importance, therefore in this paper *P. antennatus* has been described as *P. minutus* var. *antennatus* (Newstead and Sinton 1921).

#### A. DERAJAT AREA.

##### (2) Localities.

(a) DERA ISMAIL KHAN. In August, 1919, 83 specimens of this fly were caught in the bedroom of a bungalow, of these 6 were *P. papatasi* (3♂♂, 3♀) and 77 were *P. minutus* (33♂♂, 34♀♀).

In the autumn of 1919, out of 174 specimens caught in a bedroom of another bungalow, 8 were specimens of *P. papatasi* (2♂♂, 6♀♀), 152 were *P. minutus* (118♂♂, 34♀♀) and 14 were *P. minutus* var. *antennatus* (11♂♂, 3♀♀).

During March, 1920, out of 106 specimens caught mostly in a cowshed, 92 were *P. papatasi* (40♂♂, 52♀♀), 10 were *P. minutus* (3♂♂, 7♀♀) and 3 were *P. minutus* var. *antennatus* (2♂♂, 1♀) and 1 was *P. sergenti* (♂). Of 4 other specimens caught in a tent during the same month 1 was *P. papatasi* (♂) and 4 were *P. minutus* (1♂, 3♀♀).

During the first half of April, 1920, 135 specimens were caught in the cowshed mentioned above, of these 109 were *P. papatasi* (35♂♂, 74♀♀), 23 were *P. minutus* (15♂♂, 8♀♀) and 3 were *P. minutus* var. *antennatus* (1♂, 3♀♀).

Thirteen specimens were collected in the same shed between the middle of April and the middle of May, and were identified as *P. papatasi* (3♂♂, 10♀♀).

During this last period 23 specimens were caught in the bedroom of a bungalow, of these 13 were *P. papatasi* (6♂♂, 7♀♀), 8 were *P. minutus* (6♂♂, 2♀♀) and 2 were *P. minutus* var. *antennatus* (2♂♂).

During the same period, 68 specimens were collected from tents and of these specimens 15 were *P. papatasi* (4♂♂, 11♀♀), 49 were *P. minutus* (36♂♂, 13♀♀), 3 were *P. minutus* var. *antennatus* (1♂, 2♀♀) and 1 was *P. sergenti* (1♂).

These specimens may be tabulated as follows:—

Period.	<i>P. papatasi</i> .		<i>P. minutus</i> .		<i>P. minutus</i> var. <i>antennatus</i> .		<i>P. sergenti</i> .	
	♂	♀	♂	♀	♂	♀	♂	♀
August, 1919	3	3	33	34	..	..	..	..
Autumn, 1919	2	6	118	34	11	3	..	..
Spring, 1920	89	154	61	33	6	5	2	..
TOTAL	257		313		25		2	

(b) TANK.—From the end of August to the middle of September 1919, out of 18 specimens caught in the Officer's Rest Camp, 6 were *P. papatasi* (1 ♂, 5 ♀), 9 were *P. minutus* (3 ♂, 6 ♀) and 3 were *P. minutus* var. *antennatus* (3 ♀). Two specimens collected in the same tents on 6th April, 1920, were *P. papatasi* (2 ♂).

(c) HATHALA.—On 29th August, 1919, two specimens of *P. papatasi* (1 ♂, 1 ♀) and 3 of *P. minutus* (3 ♀) were caught here. One specimen of *P. papatasi* (1 ♀) was caught on 6th April 1920.

(d) In a stable by the roadside, about 6 miles from Dera Ismail Khan on the road to Tank, 4 specimens of *P. sergenti* (3 ♂, 1 ♀) were found on 6th April, 1920.

(e) JATTA POST.—At this post a specimen of *P. papatasi* (1 ♂) was obtained from the barracks on 7th April, 1920.

(f) MURTAZA POST.—On 1st August, 1919, 6 specimens were caught of which 5 proved to be *P. papatasi* (1 ♂, 4 ♀) and one *P. sergenti* (1 ♂). On 7th April, 1920, 7 specimens caught were all *P. papatasi* (1 ♂, 6 ♀).

(g) KHIRGI CAMP.—Two specimens of *P. minutus* (2 ♀) were found here on 30th August, 1919.

(h) SAGER.—One specimen of *P. papatasi* (1 ♂) was collected on 14th March, 1920.

#### B. BANNU AREA.

(a) BANNU CANTONMENT.—About the middle of August, 1919, 58 specimens of 'sandflies' were caught in the hospital at Bannu. On examination these were found to be 5 specimens of *P. papatasi*.

(4 ♂♂, 1 ♀), 74 specimens of *P. minutus* (37 ♂♂, 37 ♀♀) and 9 specimens of *P. minutus* var. *antennatus* (2 ♂♂, 7 ♀♀).

(b) IDAK FORT.—Two specimens of *P. minutus* (2 ♂♂) and 1 specimen of *P. papatasi* (1 ♀) were caught in August, 1919.

### (3) Seasonal Prevalence.

In the Waziristan area in the places situated on the plains 'sandflies' began to appear about the middle of March and were present in fairly great numbers until the middle of October.

From the above records *P. papatasi* seems more prevalent in the early part of the year and *P. minutus* in the autumn, but further observations are necessary to confirm this.

In the autumn of 1919, both *P. papatasi* and *P. minutus* were seen as late as 14th November in the bedroom of a bungalow in Dera Ismail Khan, while in Bannu a specimen of *P. papatasi* was seen on 29th October.

In the spring of 1920, in Dera Ismail Khan, in the cowshed referred to above no 'sandflies' could be found on 4th March, but on 8th, 5 specimens of *P. papatasi* were captured, on the next day they were more numerous, and a week later they were very numerous. From this time onwards hundreds of 'sandflies' could be seen on the walls in the dark corners of the shed.

At Bannu, the earliest record was on 25th March, but they were probably present before that date, as some cases of Pappataci Fever had occurred before that day.

Within a few days of the first recorded appearance of the phlebotomi at Dera Ismail Khan cases of Pappataci Fever began to occur.

### (4) Local Distribution.

From the above records it will be seen that certain species of *Phlebotomus* were common in some situations, while others were rare. In the cowshed at Dera Ismail Khan the majority of specimens caught were *P. papatasi*, while the specimens caught in the bungalows in Dera Ismail Khan and Bannu were mostly *P. minutus*. This was also the case in the tents in Dera Ismail Khan. The four specimens caught in the stable on the Tank Road were all *P. sergenti* and only 3 other specimens of this species were caught in the area.

It is therefore necessary to examine many different buildings and situations in the same locality to get an accurate idea of the different species present.

(5) *Geographical Distribution of Phlebotomus sergenti and the Oriental form of cutaneous Leishmaniasis.*

For some time flies of the genus *Phlebotomus* have been suspected to be the carriers of 'Oriental Sore,' but no definite evidence has yet been found to prove the connection.

The distribution of this genus is very widespread, while that of 'Oriental Sore' is more or less localised to definite localities. It is therefore possible that one species only of this fly may be responsible for the carriage of the disease.

The commoner 'sandflies,' such as *P. papatasi*, *P. minutus*, etc., occur in many places where 'Oriental Sore' is not found, which are points against these species being the carriers of the disease. Of course, such evidence does not negative these species as carriers, because local endemicity might depend on various local conditions, such as temperature, humidity, presence of human sources of infection, etc.

On the hypothesis that the carriage may be due to one species of *Phlebotomus*, it is interesting to note the recorded geographical distribution of *P. sergenti*.

This fly has been recorded from Algeria. Newstead (1920) records it from Mesopotamia and states that *P. caucasicus*, Marzinowsky, 1917, found in the Caucasus at Tiflis is this species.

The specimens here recorded are the first which have been described from India, and Professor Newstead identified a number of specimens of *Phlebotomus* which I sent him from Meshed in North-East Persia as all being this species.

As Algeria, Mesopotamia, the Caucasus, Persia and the North-West Frontier of India are all places where 'Oriental Sore' is prevalent, it is a curious coincidence that so far *P. sergenti* has only been recorded in these places.

On the supposition that *P. sergenti* may be a possible carrier of 'Oriental Sore,' it will be interesting to see if this species can be found in other localities where this disease is prevalent.\*

\* Since writing the above I have had the opportunity, through the kindness of Major F. W. Cragg, I.M.S., of examining some *Phlebotomus* collected by Dr. J. L. Matter at Lahore, Punjab, India, in 1918, and among these I have found two specimens of *P. sergenti* (♂). This is of interest, as 'Oriental Sore' occurs in Lahore.

I am deeply indebted to Professor Newstead, F.R.S., for allowing me to work in his laboratory at the Liverpool School of Tropical Medicine, and also for the identification of a large number of specimens. My thanks are also due to Miss A. M. Evans, M.Sc., for much assistance while working at Liverpool.

#### ANOPHELINI.

##### (1) *Varieties of Anophelini found.*

In the Derajat area specimens of *A. pulcherrimus*, *A. culicifacies*, *A. stephensi*, *A. rossi*, *A. fuliginosus*, *A. funestus* var. *listoni*, *A. turkhudi*, *A. rhodesiensis* and *A. mursei* were found.

In the Bannu area specimens of *A. pulcherrimus*, *A. rossi*, *A. culicifacies*, *A. funestus* var. *listoni*, *A. maculipalpis*, *A. stephensi*, *A. rhodesiensis* and *A. turkhudi* were collected.

#### A. DERAJAT AREA.

##### (2) *Localities.*

(a) DERA ISMAIL KHAN CANTONMENT.—In 1919, a specimen of *A. culicifacies* was caught on 22nd March, and anopheline larvæ were first found on 5th April.

From 22nd March until 4th May the number of anophelines caught and identified was 257, of which the numbers and species are given in the following table:—

<i>A. culicifacies</i>	..	..	..	230
<i>A. stephensi</i>	..	..	..	15
<i>A. fuliginosus</i>	..	..	..	4
<i>A. pulcherrimus</i>	..	..	..	8
TOTAL				257

I am indebted to Major Flowerdew, I.M.S., for supplying me with the above information.

During the summer and autumn of 1919, I collected a number of anophelines, but unfortunately these were destroyed by ants, so I have no information as to the relative frequency of the various species. Specimens of *A. pulcherrimus*, *A. stephensi*, *A. culicifacies*, *A. turkhudi*, and *A. fuliginosus* were caught, and of these the first mentioned was the most numerous.

Anopheline larvæ were seen in a pool in the bed of the Indus River on 26th December, 1919, and numerous culicine larvæ were found in a water-channel about the same time.

From this time onwards until 2nd April, culicine larvæ began to be more numerous although no anopheline larvæ could be found, but from some nymphs collected on 20th March, a specimen of *A. stephensi* and one of *A. pulcherrimus* hatched out.

On 4th March, 2 specimens of *A. culicifacies* were caught in a cowshed, and the first specimen of *A. pulcherrimus* was caught on 9th March.

During March, 25 anophelines were collected, of which 18 were *A. culicifacies*, 6 were *A. pulcherrimus* and 1 was *A. turkhadi*.

Out of 112 specimens of anophelines caught from 1st to 22nd April, 1920, 5 were *A. stephensi*, 93 were *A. culicifacies*, 9 were *A. pulcherrimus*, 3 were *A. turkhadi* and 2 were *A. fuliginosus*.

During April, anopheline larvæ were found in many situations and adults hatched out from these larvæ were *A. culicifacies*, *A. pulcherrimus*, *A. stephensi* and *A. turkhadi*, of which the former were the more numerous.

(c) TANK. In the summer and autumn of 1919, numerous anophelines were present in the tents in the Officer's Rest Camp. The most numerous species was *A. pulcherrimus* but specimens of *A. stephensi*, *A. culicifacies* and *A. rossi* were also present.

On 7th March, 1920, 2 specimens of *A. stephensi* were caught in the same tents, and at the beginning of April, 7 other specimens of its species were collected.

On 5th April, numerous anopheline larvæ were found near the ice factory and in pools  $2\frac{1}{2}$  miles along the Dera Ismail Khan Road. These larvæ hatched out into adult *A. stephensi*.

(c) MURTAZA. During August, 1919, specimens of *A. pulcherrimus*, *A. stephensi*, *A. culicifacies*, *A. rossi* and *A. rhodesiensis* were caught in the barracks in this post.

On 6th April, 1920, 9 specimens of *A. culicifacies*, and 1 each of *A. pulcherrimus*, *A. stephensi* and *A. narsei* were collected there.

(d) JALIA. At the beginning of August, 1919, anophelines were very numerous in the barracks and specimens of *A. pulcherrimus*, *A. stephensi*, *A. culicifacies*, *A. funestus* var. *lestani*, *A. rossi* and *A. rhodesiensis* were caught.

A specimen of *A. rhodesiensis* caught here on 2nd September laid eggs on 4th, which hatched into larvæ on the night 5th-6th, became pupæ on 14th and developed into adults on 16th.

On 6th April, 1920, 3 specimens of *A. culicifacies* and a specimen of each of *A. pulcherrimus* and *A. stephensi* were collected.

(e) KAUR BRIDGE.—During the hot weather of 1919, a few specimens of *A. pulcherrimus* and *A. stephensi* were caught.

(f) MANZAI.—In the autumn of 1919, a few specimens of *A. pulcherrimus* were collected, and on 16th January 1920, a specimen of *A. turkhudi* was caught.

(g) KHIRGI.—During the hot weather of 1919 *A. pulcherrimus* was fairly numerous in this camp.

(h) HATHALA.—In the autumn of 1919 specimens of *A. pulcherrimus* and *A. rossi* were caught there.

(i) POTAH.—One specimen of *A. culicifacies* was collected there at the beginning of April, 1920.

(j) KULACHI.—One specimen of *A. culicifacies* was caught at this place during the hot weather of 1919.

(k) DRABAND.—Two specimens of *A. stephensi* and 1 of *A. culicifacies* were caught there in March, 1920.

(l) SAGGU.—Numerous specimens of *A. rossi* and *A. culicifacies* were found in a stable at this place on 28th September, 1919.

(m) YARIK.—On the same date a few specimens of *A. rossi* and *A. fuliginosus* were caught.

## B. BANNU AREA.

(a) BANNU CANTONMENT.—In 1917, the first anophelines were seen in Bannu on 7th April, when 1 specimen of *A. stephensi* and 4 of *A. maculipalpis* were caught.

From some nymphs found on 11th April, a large number of *Theobaldia spathipalpis* and 1 *A. maculipalpis* hatched out.

During the month of April 173 anophelines were caught and identified as shown in the table below:—

Sex of mosquito.	<i>A. stephensi</i> .	<i>A. funestus</i> var. <i>listoni</i> .	<i>A. maculi-</i> <i>pulpis</i> .	<i>A. turkhudi</i> .	<i>A. pulcher-</i> <i>rimus</i> .	Total.	Total per cent.
♀ ..	11	22	25	2	1	61	35
♂ ..	16	27	60	8	1	112	65
TOTAL .	27	49	85	10	2	173	100
TOTAL PER CENT	15.6	28.3	49.2	5.8	1.1	..	..

It was found that a number of the mosquitoes were infested with mites which were clinging to the legs, thorax, or abdomen. The number on a mosquito varied from 1 to 5. The incidence on the various species of anopheline was as follows :—

Sex of mosquito.	<i>A. stephensi</i> .	<i>A. funestus</i> var. <i>listoni</i> .	<i>A. maculipalpis</i> .	Total.
♂	0	2	0	2
♀	2	0	1	3
TOTAL	2	2	1	5

On 1st May, a number of larvæ of *A. stephensi* were found in conjunction with the larvæ of *T. spathipalpis* and from this time onwards anopheline larvæ were very common, especially in the Kurram swamp.

The anophelines caught during May were as follows :—

Sex of mosquito.	<i>A. stephensi</i> .	<i>A. funestus</i> var. <i>listoni</i> .	<i>A. maculipalpis</i> .	<i>A. turkheadi</i> .	Total.	Total per cent.
♀ ..	11	12	11	1	35	25
♂ ..	31	31	38	4	104	75
TOTAL	42	43	49	5	141	..
TOTAL PER CENT	34.7	31.9	29.9	3.5	..	..

The species infested with mites were :—

Sex of mosquito.	<i>A. stephensi</i> .	<i>A. funestus</i> var. <i>listoni</i> .	<i>A. maculipalpis</i> .	Total.
♂ ..	3	0	0	3
♀ ..	1	3	1	5
TOTAL ..	4	3	1	8

Of the anophelines examined during this month 5.6% showed an infestation with mites.

(b) IDAK FORT.—Height about 2,140 feet. In the autumn of 1919, specimens of *A. stephensi*, *A. culicifacies*, and *A. funestus* var. *listoni* were found in scanty numbers.

(c) KHAJURI POST.—Height about 2,080 feet. Very numerous anophelines could be caught in this post during the autumn of 1919 and many anopheline larvæ were found in the numerous springs in the vicinity. Specimens of *A. rossi*, *A. rhodesiensis*, *A. stephensi*, and *A. pulcherrimus* were identified there.

(d) SAIDGI POST.—Height about 1,775 feet. During the hot weather of 1919, specimens of *A. stephensi*, *A. culicifacies*, *A. rossi* and *A. rhodesiensis* could be found in large numbers.

(e) SHINKI POST.—In September, 1919, one specimen of *A. stephensi* and one specimen of *A. funestus* var. *listoni* were caught at this post. The occurrence is of interest as the post is situated on the top of a barren, stony hill almost devoid of vegetation about 700 feet above the river, which is the nearest water. There was no chance of these mosquitoes having bred locally as the post had been burnt down some months previously, so local water-receptacles could be excluded as breeding places. The only solution of their presence seems to be that they were carried there by the wind.

(f) MIRANSHAH FORT.—Height about 3,050 feet. In June, 1917 specimens of *A. stephensi*, *A. turkhudi* and *A. funestus* var. *listoni* were caught.

(g) At Dardoni Cantonment in the autumn of 1919, one specimen of *A. stephensi* was caught.

(h) At Gambila between Bannu and Dera Ismail Khan a specimen of *A. rossi* was found in a tent on 29th December, 1919.

#### SUMMARY.

From the above it will be seen that *P. papatasi*, *P. minutus*, *P. minutus* var. *antennatus* and *P. sergenti* occur in the Waziristan area of the North-West Frontier Province.

The anophelines recorded are *A. turkhudi*, *A. culicifacies*, *A. funestus* var. *listoni*, *A. rhodesiensis*, *A. fuliginosus*, *A. rossi*, *A. maculipalpis*, *A. stephensi*, *A. pulcherrimus* and *A. nursei*. From this list it will be seen that the anopheline fauna of this area is practically the same as that described by me (Sinton, 1917) from the Kohat District which lies just north of the Bannu District. The only species not recorded being

*A. willmori*, but a more careful search will probably find this species in the Waziristan area.

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## REPORT ON ELECTROLYTIC CHLOROGEN (E.C.).

BY

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AND

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[Received for publication, July 13, 1920.]

IN consequence of the outbreak of a very serious epidemic of cholera in the district surrounding Pusa in the hot weather of 1918, an attempt was made to carry out sterilization of wells both in the estate and in surrounding villages. The difficulty of obtaining adequate supplies of reliable antiseptic led one of us to enquire into the possibility of producing hypochlorite solutions in the laboratory to use for this purpose. After a certain amount of work had been done it was suggested that this line of enquiry, if successful, might be of value to the Army Department in India, and subsequent research was carried out with this object and with the sanction and encouragement of Army Headquarters in Simla. The work was greatly helped by the assistance of Captain W. Hodgkinson, R.E., put on deputation for the purpose, whose special knowledge as an Electrical Engineer and experience in Mesopotamia proved invaluable throughout the enquiry. Before Captain Hodgkinson joined this laboratory much trouble was experienced owing to the absence of proper apparatus for standardizing the electrical conditions under experiment, but this officer obtained and fitted sets of controlling and measuring instruments, enabling all experiments to be carried out under completely controlled conditions.

*Objects of the enquiry.*—The principal objects of the enquiry were to determine the possibility of preparing in India, by the use of simple

apparatus, solutions of hypochlorites of sufficient stability to allow of their use for sterilizing drinking water under field conditions or otherwise. Simplicity of apparatus was aimed at, because it was intended that the method should be suitable for use by any medical officer without any special knowledge either of electrical practice or chemical theory and in any situation where electric current might be available. Stability was insisted on by the Stores Department of the Indian Medical Service, as from their point of view this was naturally of great importance. It may be said at once that this feature of the case was kept in view as of primary importance throughout the enquiry, although taking into consideration the facts that the antiseptic action of hypochlorites depends upon the readiness with which they give up their available chlorine, and their well-known instability in solution, it was early recognized that such stability as might be obtained could only be of a comparative order. Keeping this in mind it was our aim to ascertain to what extent hypochlorite solutions could be stabilized either during or after preparation, and to devise apparatus for producing them—of such simplicity as to allow of its use wherever electric current was available, the apparatus itself being so arranged as to produce a standard strength of solution under fixed conditions from simple materials obtainable in any bazaar in India. As compared with the issue from store of completely stable solutions, if such could be obtained, this arrangement would possess the advantage of requiring less transport and no testing of strength for each freshly prepared lot, as is the case with "bleach." The actual value of this method would then depend upon the relationship between the stability of the solution and the distance between the points of production and of use.

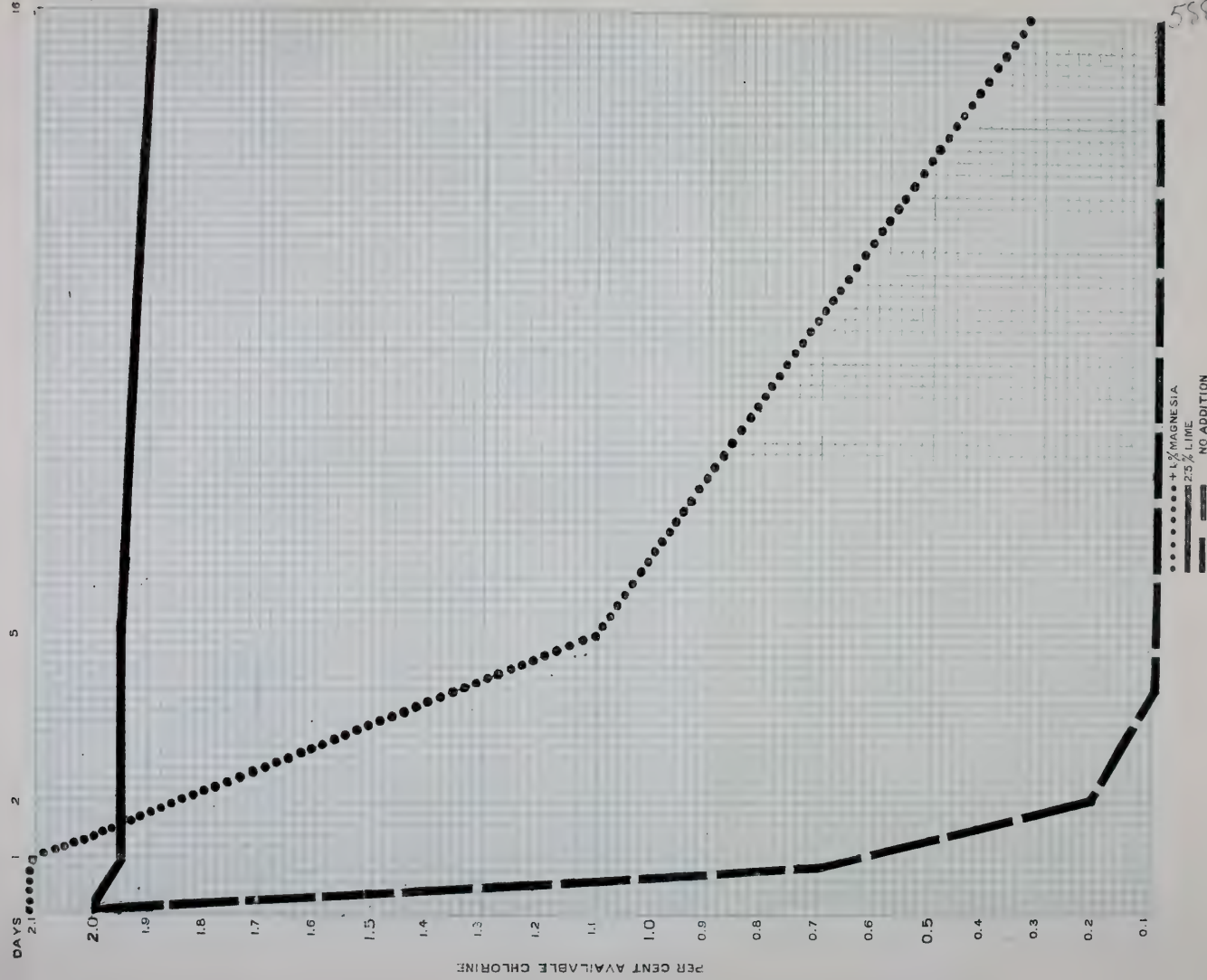
*Alternative methods of preparing hypochlorite solutions.*—Hypochlorite solutions can be readily prepared either by direct chemical methods from bleaching powder or by electrolysis of brine or of other solutions of chlorides. The electrolytic method was first adopted in this enquiry, because of the availability at Pusa of electric current and the difficulty of obtaining bleaching powder under war conditions; subsequent investigation led to the conclusion not only that the relative availabilities of salt and bleaching powder in India gave the electrolytic method a very great advantage, but that solutions thus prepared compared favourably in other ways with those made either directly from "bleach" as in ordinary I. M. S. practice, or sophisticated in various ways aiming at stabilization. A certain amount of work was done on

chemical preparation of hypochlorite solutions using chlorine gas obtained by electrolysis in a diaphragm cell, but practically the whole of the enquiry was limited to investigation of the possibilities and optima of the electrolytic method.

The electrolysis of brine has been commonly used for many years in Europe both as a source of chlorine in chemical manufactures and of bleach liquors containing hypochlorites. Numerous patents have been taken out for various forms of apparatus for these purposes, and in some cases claims have been made for stability in the product, but in these the nature of the stabilizing agent has generally been concealed, and this reticence has also been largely in evidence even where the addition of chemical reagents has had for its main object the attainment of higher concentrations of available chlorine than are possible without them. Several forms of electrolytic cells are now on the market intended mainly for the production of hypochlorite solutions for bleaching purposes or occasionally for antiseptic use. These, however, produce solutions of low concentration and stability, suitable and intended only for immediate use. As will be shown later in this report such cells are unsuitable for the production of stable solutions of higher concentration, mainly on account of the absence of any cooling arrangement in their design. Such cells normally produce solutions of about 0.5 per cent concentration of available chlorine, whereas for storing, transport, and use in water sterilization, concentrations of some four or five times this amount are required for convenience in handling.

The electrolysis of brine results in the production of sodium hypochlorite in quantities which vary in accordance with the conditions of the experiment. One of the most important factors is temperature, as, when this is allowed to rise about 37° C., chlorates are formed at the expense of the hypochlorites and consequently with reduction of the percentage of available (*i.e.*, useful in the antiseptic sense) chlorine in the resulting solution. Not only is it necessary to keep the temperature well below this limit for this reason, but we find that there is a decided tendency towards parallelism between low temperature during electrolysis in the electrolyte and high available chlorine content in the resulting solution. This is no doubt due partly to avoidance of excessive loss of chlorine as gas and partly to absence of formation of chlorates and other products. Owing to the resistance of the brine, heating occurs in the neighbourhood of the electrodes and provision has to be made to keep this down to a minimum; the absence of provision for cooling in the various commercial

Expt. I, Curve A



558<sup>a</sup>



forms of cell now on the market prevents high concentrations being obtained, whereas one of the objects of this enquiry was to devise a form of cell in which cooling could readily be carried out during electrolysis. A brief description of the various types of cell experimented with is given as an appendix to this report; it may be pointed out here that our efforts were strictly limited by the apparatus and materials available and the difficulties incidental to war conditions.

Previous experiments by Muspratt and Smith, Forster and Jorre, Lunge and Landolt, Sunder, Mulhouse, and others have demonstrated certain limits in obtaining concentrations of available chlorine as hypochlorite by electrolysis of brine. One of the limiting factors is the rapid lowering of current efficiency as concentration proceeds; thus, at the commencement of electrolysis as much as 90 per cent of the theoretical quantity of hypochlorite corresponding to the electrical energy used is obtained. This rapidly falls off, however, so that when a concentration of about the order of 2.5 per cent available chlorine is reached, further electrolysis only yields very slight increase in concentration per unit of electric current consumed. (Experiments Nos. VI A.

We have found at Pusa not only that 2.3 per cent concentration of available chlorine is about the maximum obtainable with anything like economy of current consumption (Experiments Nos. II, VI A and XIII A), but also that stability rapidly falls off in higher concentrations than this. As a result of numerous experiments we are now of opinion that a concentration of about 2.5 per cent available chlorine is the optimum to aim at, both for reasons of economy in production and stability of the product.

*Stability*.—As is already well known, hypochlorite solutions produced simply by electrolysis of brine possess no stability, losing practically the whole of their available chlorine in a comparatively short period of time; this varies inversely as the temperature, but at plains hot weather temperatures may be as little as 24 hours (Experiment XIV). It is therefore necessary to discover how far this instability may be overcome by alteration of the electrolytic conditions. Such alteration may be either considered from the chemical or from the electrical point of view, the latter being taken to include the physical factor of low temperature. Various substances have been tried for this purpose by previous experimenters; of these the salts of magnesium, generally the chloride, appear to have been most frequently utilized on a commercial scale. In the case of such additions, the general aim has been to produce a compound

hypochlorite of higher stability in solution than the simple sodium hypochlorite.

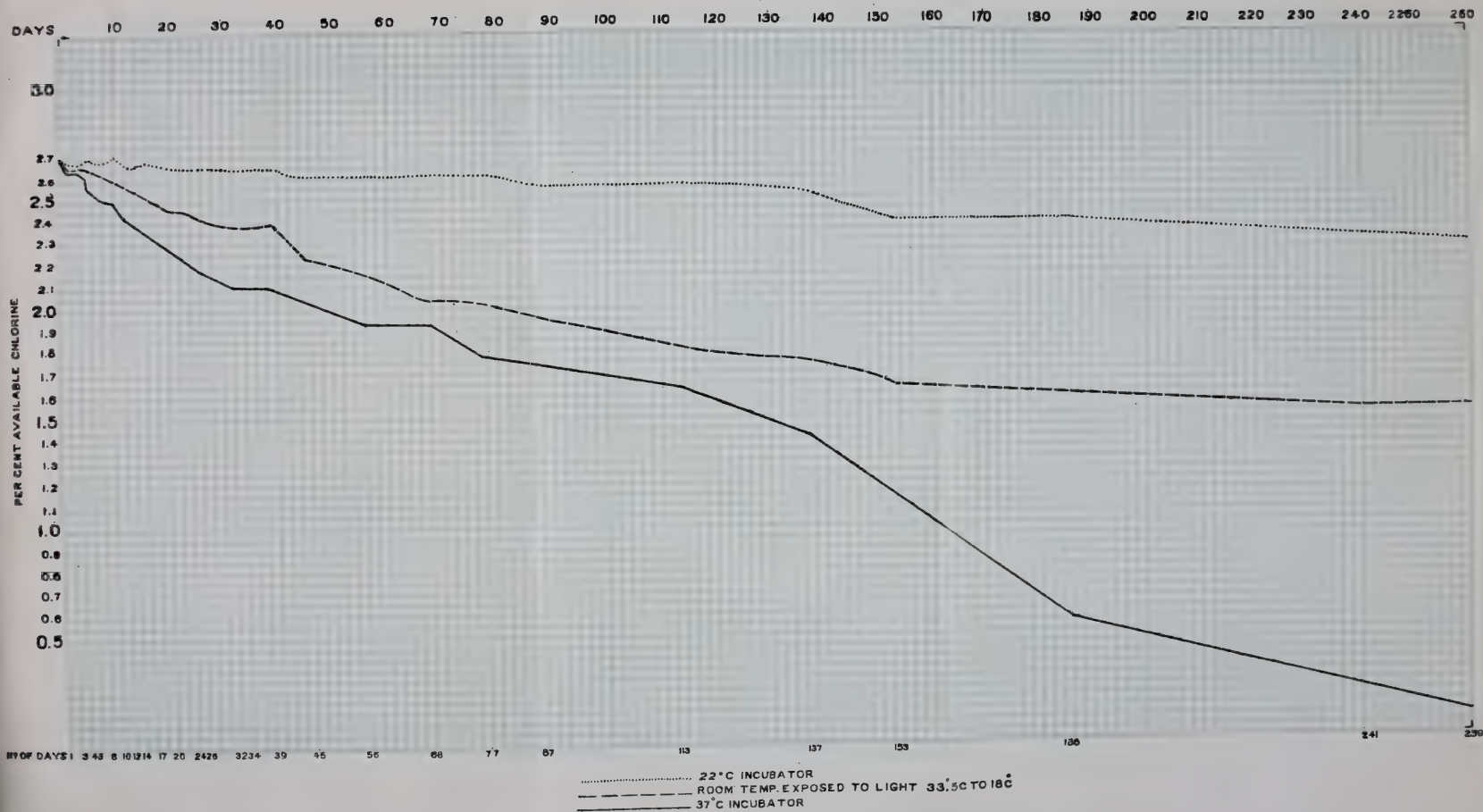
Both the well-known Hermite fluid and that produced and distributed by the Poplar (London) municipal authorities for disinfecting purposes contain magnesia, and this salt and the chloride were both experimented with at Pusa; it was found, however, that although the addition of magnesia to the brine gave increased stability and allowed of higher concentrations of available chlorine being obtained than was the case with brine alone, such addition did not ensure stability of any high or useful order (Experiment No. I). The use of magnesia in connection with other stabilizing substances tested has not been found to have any marked effect on either concentration or stability (Experiments IX E and IX F). It may be said at once that the principal conclusion arrived at as a result of our experiments was that the high temperatures obtaining in the plains of India make it necessary to revise all ideas as to the stability of hypochlorite solutions prepared, used, and possibly found reliable in other cooler countries. It may be pointed out, for instance, that a certain proprietary article made in England, and now being largely advertised as a stable antiseptic solution, on examination in this laboratory was found to contain available chlorine in concentrations which varied in two samples from 1 per cent in one case to 0.3 per cent in the latter, and this during the cold weather (February 1920). (*Cf.* Curve B—Bulk 16. Stability.)

The addition of calcium chloride has been prescribed as favourable to formation of hypochlorite solutions by electrolysis (Martin. *Industrial Chemistry*, Vol. I, p. 397) and by chemical methods. We have not found any increase of stability or concentration due to its use.

The addition of caustic soda or potash is frequently recommended to increase stability; that is either on the grounds of securing alkalinity and so avoiding accumulation of free hypochlorous acid, or of promoting the formation of soluble carbonates of potash or soda in preference to insoluble and consequently precipitable calcium carbonate as a result of exposure to air. In the latter event, it is assumed that a continuous action would set up resulting in the complete loss of all available chlorine. It appears to us that this theory involves the assumption that such sodium or potassium carbonate as might be formed would remain as a protective surface layer in virtue of its solubility, but this property would also naturally lead to its diffusion; moreover, it is not contemplated

# STABILITY OF E.C. (BULK 16) AT DIFFERENT TEMPERATURES

59%  
Curve B





to expose the solution to indefinitely large volumes of air. Notwithstanding the very general assumption as to the stabilizing effect of caustic soda or potash, we have found no such action in our experiments with the electrolysis of brine (Experiment XV A). It is, on the other hand, possible that, in the case of solutions made from bleaching powder the addition of caustic alkalies might tend to stabilize the product by removing salts of iron existing as impurities in the bleach, the presence of which is well known to cause rapid decomposition of hypochlorites by catalysis. This is a point to be kept in view in making comparative tests of solutions of "bleach" and of electrolyzed brine, as the former will vary largely in stability in accordance with the presence or absence of iron salts in the original bleaching powder. It is interesting to note that we have not found iron in any of the brine solutions made from numerous samples of bazaar salt in this laboratory.

*Borax*.—The use of this salt has been recommended as a stabilizer, it has some effect, but only in low concentrations of less than 1 per cent available chlorine. (Experiments XV and XV A.) It may here be pointed out that a 3 per cent salt solution, electrolysed to a strength of 0.5 per cent available chlorine to which 0.5 per cent of borax is added, may be used as Dakin's solution (Cutler and Hubbard, *Journal of Biological Chemistry*, Vol. XXXVII, No. 4).

*Lime*.—The addition of lime as hydrate was found to produce a stabilizing effect of a high order as compared with that of any other substance tested. It will be seen, by reference to the tables of experimental results in the appendix that the stability obtained by its use, although not by any means absolute, is yet very much higher than that so far obtained by any other method. It appears probable that in the presence of sufficient excess of lime a considerable proportion of the hypochlorite formed is calcium rather than sodium hypochlorite, or at any rate that calcium hypochlorite is the end product of various chemical reactions taking place both during and after electrolysis. That such changes continue after electrolysis may be inferred from our observation that stabilization may be secured by the addition of the lime after completion of electrolysis (Experiments XVIII A and B) but that they also occur during this operation is evident from the fact that addition of lime before, or during, electrolysis allows of the attainment of higher degree of concentration of available chlorine. (Experiments XVI and XVIII.) In view of the greatly increased stability obtained by the use of lime numerous experiments were carried out to determine, if possible, the best method of utilizing the

substance. These experiments generally aimed at variations in the amounts used, and of the method of addition. The addition of lime subsequent to electrolysis was also used to obtain a clear product. Some success was obtained by the use of potassium chromate as a stabilizer, but it did not seem advisable to introduce such a substance even in negligible quantities into a water sterilizer.

Another method of increasing stability is to introduce a readily diffusible colloidal substance, which may retard decomposition of the hypochlorite by its physical properties, this principle is made use of in connection with the preparation of other compounds such as hydrazine, but very little is known of the actual reactions involved and the method may be described as an empirical one, although like many others similarly obscure in their action, it is of undoubted value. Of the substances of this class tried by us at Pusa, sodium rosinate was found to be the most efficient as a stabilizer in conjunction with lime (Experiments IV A and C).

In view of the absence of knowledge as to the actual mode of action of such substances and the empirical nature of such experiments in this direction as we have carried out, it would appear advisable to extend these experiments considerably in likely directions with the intention, not only of discovering a means of obtaining higher stability but of securing higher concentration. It has been pointed out above that such higher concentration so far seems to involve not only loss of stability but of efficiency in the use of the electric energy consumed. The aim of further experiment should therefore be to combine high stability and concentration without undue consumption of current.

*Concentration of Electrolyte.*—The strength of brine solution used is of importance as it is found that it is necessary to employ strong solutions (20 per cent—25 per cent NaCl) in order to obtain any degree of concentration without undue waste of electrical energy (Experiment V). At the same time the higher conductivity of strong brine reduces the amount of heating due to the resistance of the electrolyte and therewith renders it easier to avoid formation of chlorate. In commercial electrolysis of brine on a large scale for production of bleach liquor the actual concentration of salt employed is frequently as low as 10 per cent, but this is utilized so as to effect economy in salt rather than in electric current, as high concentrations of available chlorine are not aimed at, less than 1 per cent being generally required. In order to obtain concentrations of 2.5 per cent to 3 per cent, such as we have aimed at,

we find it necessary to use brine of at least 20 per cent concentration: this also serves to reduce the tendency to heating.

*Electrical conditions.*—We do not propose in this report to enter into any elaborate discussion of electrolytic theory nor to discuss in any detail the economic side of the expenditure of electrical energy in the production of hypochlorite solutions. We shall content ourselves with giving in the tables of experimental results the number of ampere hours of the electricity consumed as actually measured by a meter during the running of the experiment: from this it will be easy to make any necessary calculations as to the cost of production of hypochlorite solutions by our method with reference to local supplies of current at various rates. The cost of bazaar salt required can be calculated by reckoning on the use of a 25 per cent solution. The cost of the additions of lime and sodium rosinate is negligible, owing to the small quantities used.

*Voltage.*—To the "polarization" voltage theoretically required for the splitting up of NaCl must be added that necessary to overcome the combined resistances of the electrolyte, and of the electrodes and connections of the cell: in actual practice we find 6–8 volts per cell approximately necessary, so that with an ordinary lighting current of 110 volts 12 to 18 cells can be used in series and at an efficiency of 50 per cent each ampere hour will render available 6½ grams of chlorine *per cell*. At lower concentrations such as required for Dakin's solution an efficiency of as much as 75 per cent may be expected. The capacity of the cells will of course depend upon the current available, and this in a lighting circuit will again depend on the current-carrying capacity of the wiring which may vary in an ordinary house or office from 1 amp. up to 10 amps. or more. With a separate generating plant such as would be available on a river steamer or at a base hospital, the amount of current available for electrolysis would depend upon the output of the generator and the other duties of the installation. In any case, however, the actual current required is comparatively little, and the smallest generator would only require a few hours' running overtime to produce enough hypochlorite solution for ordinary requirements. Thus a 5 Kw. generating plant suitable for X-ray work and operation theatre illumination would produce 4 gallons of hypochlorite solution of 2.5 per cent Cl strength (referred to hereinafter in this report as E. C.) per hour, this quantity being sufficient to sterilize 80,000 to 160,000 gallons of water. It will be seen therefore

that, with a stability period in the hot weather of six weeks, it should be possible to produce this solution at points well within the range of transport permitted by such a period of stability. The same plant would produce approximately 30 gallons of Dakin's solution of 0.5 per cent  $\text{Cl}_2$  strength per hour. Alternatively, a complete outfit for field work, comprising  $1\frac{1}{4}$  Kw. dynamo, mule gear and cells, could be made to form a load for four small mules and weigh less than 600 lbs. and could be operated by two mules. This would produce sufficient E. C. to sterilize, say, 20,000 gallons of water per hour.

*Current.*—In commercial electrolytic practice economy is effected by the use of large currents of low potential, the latter being kept down to the minimum necessary to overcome the resistances in the cell. In the production of hypochlorites the chief point in the electrical conditions necessary for efficiency is the provision of a high current density, *i.e.*, the ratio of current to electrode surface; an efficient ratio is found to be about 1 ampere per sq. cm. of electrode surface. Lower current densities tend to increase the secondary reactions occurring in the cell, such as result in the liberation of undue quantities of hydrogen with consequent reduction of the hypochlorite formed.

*Electrodes.*—The expense of platinum iridium and platinum electrodes made it necessary to work mainly with carbon electrodes. Much trouble was experienced with these owing to their varying composition and the readiness with which they are attacked both by oxygen, hypochlorite and chlorate. Apart from the rapid destruction of the electrodes, necessitating replacement, not only is the formation of hypochlorites interfered with by this action, but the stability of the product is lowered owing to the catalytic action of the finely divided carbon particles resulting from their disintegration (Experiment). These can be removed to a great extent by settling and decantation, but in every case the stability of E. C. produced from platinum electrodes was higher than that of E. C. from carbon ones. Later, in the course of this enquiry, we were able to obtain a few samples of Acheson graphite specially made in America for electrolytic work. This material approaches very nearly to the efficiency of platinum for this purpose and, as it is cheap, being made on a very large scale by the Acheson Co., its use in place of platinum is indicated.

*Forms of cell experimented with.*—It may be said at once that no finality has been arrived at in this direction, although more than one type of cell has been devised capable of producing E. C. of standard

concentration and stability under ordinary conditions such as should be obtainable wherever current is available. A fixed standard type could readily be arrived at by the introduction of such modifications of the existing form as trial under various conditions might suggest. The chief requirement in such a cell is the provision of a method of cooling the electrolyte during electrolysis. This may be done either by air cooling or water cooling. Generally speaking the former involves circulation of the brine, whereas the latter may be obtained by running water. The former therefore either necessitates passage through the electrolyzer by gravity and the attainment of concentration by repeated electrolysis with intervals for cooling, or the same end may be obtained by the use of a pump. We find that this method involves disadvantages, owing to the high temperature reached by the brine when passed through a series of cells sufficiently long to attain the necessary concentration, or alternatively to the long period required for cooling, and the complications introduced by the use of a pump, if concentration is to be obtained by a succession of passages through the electrolyzer. Water cooling, on the other hand, is comparatively simple, especially where a pipe supply is available, although in place of this a hand-fed tank can easily be arranged. Where ice is obtainable, water cooling is naturally easily effected. It would, of course, be comparatively simple to design an apparatus for use with a pipe supply under pressure, but the latest form of electrolyzer tried is intended for use under conditions where only the flow from a hand-filled tank or cistern, with a head of a foot or two, may be available. With this form of cell the heated electrolyte rising from the gap between the electrodes comes immediately into direct contact with a cooling water tube and again with a second above it. With this arrangement we find that it is possible to keep the temperature of the brine well within five degrees of that of the cooling water, whereas with other types this difference might amount to as much as fifteen to twenty degrees centigrade. The rate of rise of temperature of the electrolyte will naturally vary *ceteris paribus* with the ratio between the current and volume of brine in the cell, so that it is difficult to obtain a high rate of electrolysis, *i.e.*, high ratio of current to electrolyte, without at the same time undergoing risk of undue rise of temperature. The rate at which electrolysis can be carried out safely will therefore depend largely upon the efficiency of the cooling arrangements, and this again will depend upon the design of the electrolyser and the temperature and rate of flow of the cooling water. Generally speaking, therefore, it may be said that

we should aim in designing our electrolyser at an appropriate mean between high rate electrolysis requiring the rapid flow of large quantities of low temperature cooling water, and the slow and prolonged action of small currents with poor cooling. Given a well-designed cooling arrangement it is merely a question of the use of a thermometer in conjunction with the variable resistance (which should form part of the apparatus and might take the same form as a fan regulator) to determine the rate of electrolysis. Where ice is available, naturally a high rate of electrolysis could be employed. It must also be remembered that in situations where cooling is difficult, owing either to shortage of cooling water or high temperature of the latter, a concentrated brine (25 per cent NaCl) must be used in order to reduce the heat due to the higher resistance of more dilute solutions.

In actual practice the type of electrolyser above referred to, of which the overall dimensions are  $14\frac{1}{2}'' \times 8' \times 17''$  and the weight about 30 lbs., working with a current of 5 amps. at 24 volts and with cooling water at  $25^{\circ}\text{C}$ ., will turn out 3.6 litres of 0.5 per cent E. C. in 6.5 hours consuming about  $\frac{3}{4}$  unit electricity. The actual outturn depends upon the number and size of the cells, and this again is determined by the current available as before explained; the electrolyser actually now in use at Pusa is made up of only four cells holding 900 cc. each; this is run once a week to produce E. C. sufficient for the sanitary requirements of the Institute and estate including the sterilization of some forty wells. The advantages of its use, as compared with that of bleaching powder for the same purposes, are numerous and evident; amongst these may be cited the following:—

- (1) Bleaching powder must be imported from Europe and loses a high and varying proportion of its content of available chlorine on the voyage. This loss continues in store in India and has been shown to amount to as much as 96 per cent of its available chlorine in eight weeks at  $98^{\circ}\text{F}$ . ( $=36.7^{\circ}\text{C}$ ) and in three weeks at  $113^{\circ}\text{F}$  ( $=45^{\circ}\text{C}$ ).
- (2) Owing to the great variation between samples of bleaching powder it is necessary to titrate every solution made therefrom to ensure the inclusion therein of a sufficient quantity of available chlorine. Owing to the difficulty of doing this in actual practice excessive quantities of bleach are frequently used with great detriment to the condition of the drinking water.

- (3) When bleaching powder has deteriorated so that it is necessary to use excessive amounts to obtain the requisite concentration of available chlorine, such deterioration generally involves the formation of soluble compounds, such as chlorates, some of which are largely responsible for the characteristic and objectionable taste of "chlorinated" water.
- (4) When "bleach" solutions are made up to standard strength their stability will be found to vary greatly in accordance with the character of the bleaching powder from which they are made, and the presence of such impurities as salts of iron and other catalysts.
- (5) The cost of imported bleaching powder when taken together with that of railway freight from the seaport compares unfavourably with that of the indigenous materials required for the manufacture of E. C. even when refined table salt is used. The cost of the electric current, when calculated merely as that of the extra fuel and lubricants required by the additional time of running the generating plant, adds a very small amount to the cost. The capital cost and recurring charges for renewals of the apparatus are comparatively small.
- (6) The advantages of independence of imported materials have been fully demonstrated by recent experience in India.

As against the above it may be pointed out that owing to the possibility of standardizing all the conditions for manufacture of E. C. this solution can be turned out of unvarying concentration so long as due attention is paid to the quantities of material used and the electrical conditions of working. It must be said, that when using crude bazaar salt in place of table salt, slight variations in stability are liable to occur, no doubt owing to the presence of impurities. The actual stability, however, is only affected in this case to a slight extent, the alteration taking the form of a more rapid falling off in concentration after passing the initial six weeks' period of comparative stability characteristic of E. C. under hot weather temperature conditions. The ability to produce practically unlimited supplies of hypochlorite solution for use in surgical dressings and for sanitary disinfection, wherever current and bazaar salt are available must give the use of the electrolytic method a considerable advantage over that of bleaching powder, which at 100° F. loses over

90 per cent of its available chlorine in eight weeks, thus necessitating the transport of large quantities of entirely inert material and continual chemical determinations of its remaining chlorine content. Notwithstanding the undoubted and recognized value of bleaching powder as an antiseptic, its use in India has been comparatively restricted, very largely by reason of the facts above cited and especially on account of the rapid loss of chlorine which it undergoes at tropical temperatures and the consequent uncertainty of its action. Local production of hypochlorite solutions of known chlorine content should go far towards extending the use of chlorine as an antiseptic, not only for sterilizing water supplies, but for surgical dressings and general sanitary purposes.

*Stability of E. C.*—Full details of tests of the stability of E. C. under various conditions of preparation and storage will be found in the Appendix to this report; the following general remarks may be made here. By the use of lime and sodium rosinate practical stability may be obtained up to about six weeks at plains temperatures ( $30^{\circ}\text{C}$ ) and to as much as one year or more on hill stations ( $20^{\circ}$ — $22^{\circ}\text{C}$ ).

Absolute stability of hypochlorite solutions is unknown owing to occurrence of hydrolysis; the relative stability period of E. C. includes an initial one of very slow loss of available chlorine followed by a second in which the rate of loss undergoes obvious acceleration. The initial or stable period varies in length on the one hand with method of preparation, and on the other with conditions of storage. With no addition of stabilizers and at plains temperatures ( $30^{\circ}\text{C}$ ) this initial period is infinitely short, and stability is nil; by the use of lime and sodium rosinate the initial period may be extended to six weeks or more at  $30^{\circ}\text{C}$  and to as much as 20 weeks or more in hill stations or in a cool incubator at  $20^{\circ}$ — $22^{\circ}\text{C}$ . It is of importance in connection with the reports as to the stability of samples of E. C. sent for testing to various laboratories, to realize the existence of this initial period of relative stability with the break in the curve occurring at plains temperatures at the six weeks' point; thus a test made of the relative stabilities of E. C. and a bleach solution made up to the same strength, will depend for its value upon taking into consideration the point in the curve at which the test began; if this test was carried out on a sample which had already approached the six weeks' point the relative stability of the E. C. would appear low as compared with one performed on a more recently prepared sample, nor would the initial dilution of the "bleach" liquor so as to produce equal concentrations in both solutions secure complete parallelism between

them. It must be further pointed out that the stability of one sample of "bleach" is no standard for such solutions in general owing to the varying occurrence of catalysts as impurities in bleaching powder. Clear indication of this fact is afforded by the very varying stability of commercial samples of sterilizers now on the market, prepared from bleaching powder.

*Antiseptic efficiency of E. C.*—Not very much experimental work on the sterilizing power of E. C. was done at Pusa, as it was taken for granted that this depended upon its content of available chlorine measured chemically (Report of the Director of Parel Laboratory, 14th May, 1919). A certain number of experiments was carried out which seemed to indicate that in this respect E. C. was strictly comparable with "bleach" solution, and those performed in the Pasteur Institute at Shillong and in the laboratory of the Sanitary Commissioner to Government of Assam appeared conclusive on this point. (Reports of Knowles and McCombie Young.)

It is of interest to note that electrolyzed brine solutions are well known to possess antiseptic efficiency of a higher order than bleaching powder solutions of equal contents of available chlorine. This is probably due to their content of hypochlorous acid, the unstable nature of which, although increasing the rate of antiseptic action of such solutions, greatly diminishes their stability, so that they can only be used effectively immediately after preparation. The addition of lime in the manufacture of E. C. prevents the formation and persistence of free hypochlorous acid. In this connection an interesting observation was made that the addition of very small quantities of ammonia to the water to be sterilized, before the addition of E. C., increased the bactericidal effect of the latter by about 100 per cent. Lt.-Col. H. Ross, I.M.S., drew our attention to the fact that this effect had been observed already.\* It would seem worth while to follow this line of experiment further.

The method of estimating the percentage of available chlorine used in this laboratory was the ordinary Pot. iodide and sodium thiosulphate routine method. This was checked by the arsenious oxide method. As confusion of ideas appears to arise occasionally as to the proper interpretation of the term "percentage of available chlorine," it might be well to point out here that this term is a conventional one arising out of the chemistry of bleaching powder manufacture; it does not

\* The use of ammonia in the chlorination of water by Joseph Ross, 1847.

imply the presence of the weight of actual chlorine stated but only half this amount as this is expressed in terms of its antiseptic capacity, which is proportional to the amount of oxygen set free coincidentally with the breaking up of the hypochlorite. A note on this point was kindly written by Mr. W. A. Davis at our request and is appended.

[The Authors desire to acknowledge the valuable assistance of Mr. C. S. Ram Ayar, B.A., Assistant to the Imperial Agricultural Bacteriologist at Pusa, in the collection of the data in the Appendix of this Report.—Ed.]

#### LIST OF EXPERIMENTS.

##### *A. Electrical.*

EXPERIMENTS	XI & XIII	..	Effect of platinum anode and carbon cathode and <i>vice versa</i> .
BULK	22	..	Comparison of platinum anode and Acheson graphite anode.
EXPERIMENT	VI A	..	Experiment showing the fall in efficiency after a certain concentration of chlorine is reached.
„	XVI	..	Effect of low current density of electrodes.

##### *B. Stability.*

EXPERIMENTS	I & IX	..	Effect of MgO on stability alone and in combination with lime.
„	Ic, XIV	..	Effect of simple electrolysis with no lime.
„	XIV A	..	Effect of potassium chromate. Comparative effect of graphite and gas carbon particles.
„	XV & XV A	..	Effect of borax as a stabilizing agent in low and in high concentrations.
BULK	16	..	Effect of temperature. Effect of dilution.
EXPERIMENT	II	..	Effect of sodium rosinate and lime.

##### *C. Concentration.*

Experiment A and Bulk	23	..	Effect of potassium chromate.
„	V	..	Comparison of 10 % and saturated solution of NaCl.
„	XVI,	..	The optimum amount of lime to be added.
„	XVIII	..	Effect of adding lime before and after electrolysis.

## EXPERIMENT I.

27-7-1918.

*Showing the comparative stability of MgO and CaO. See Chart A.*

Volume per cell.	A	B	C
	6 cells of 25 c.c. each.	6 cells of 25 c.c. each.	4 cells of 25 c.c. each.
Anode .. .. .	Arc Lamp carbon 6 mm. diameter		
Cathode .. .. .	Ditto		
Current .. .. .	Unknown		
Current density .. .. .	Unknown		
Brine .. .. .	25 % Table Salt in distilled water		
Lime, CaO .. .. .	2.5 %	Nil.	Nil.
Saturated solution of sodium rosinate .. .. .	Nil.	Nil.	Nil.
Any other addition .. .. .	Nil.	1 % MgO	Nil.
Ampere hours .. .. .	Unknown		
Final concentration of available chlorine .. .. .	1.97 %	2.12 %	1.99 %
Theoretical concentration .. .. .	.....	.....	.....
<i>Stability.</i>			
Available chlorine after —			
24 hours .. .. .	1.955 %	2.05 %	0.7 %
2 days .. .. .	1.95 %	1.80 %	0.3 %
5 „ .. .. .	1.95 %	1.40 %	0.1 %
16 „ .. .. .	1.88 %	0.33 %	.....

## EXPERIMENT II.

7-11-1918.

*Addition of Sodium Rosinate.*

	A	B
Volume per cell .. .. .	250 c.c.	250 c.c.
Anode .. .. .	Arc Lamp carbon 6 mm. diameter	
Cathode .. .. .	Ditto	
Current .. .. .	5 to 5½ amperes	
Brine .. .. .	Table salt 25 % in distilled water	
CaO .. .. .	1 %	1 %
Sodium rosinate .. .. .	Nil.	5 c.c.
Any other addition .. .. .	Nil.	Nil.
Ampere hours .. .. .		16.36
Theoretical concentration of Av. Cl .. .. .		8.93
Final concentration of Av. Cl <sub>2</sub> .. .. .	3.02 %	3.12 %
Efficiency per cent .. .. .	35.0	36.14
<i>Stability.</i>		
Av. Cl after 6 weeks .. .. .	2.87	2.99
6½ months .. .. .	1.57	1.90

## EXPERIMENT IV.

14-11-1918.

*Lime added before and after electrolysis.*

	A	B	C	D
Volume per cell .. ..	250 c.c.	250 c.c.	250 cc.	250 cc.
Anode .. ..	Arc Lamp carbon,	6 mm.	diameter	diameter
Cathode .. ..	D i t t o			
Current .. ..	5 to 5.4 amperes			
Brine .. ..	25% Table salt			
Sodium rosinate .. ..	Nil.	Nil	5 c.c.	5 c.c.
CaO .. ..	1% Nil.	1% Nil.		
Any other addition .. ..				
Ampere hours .. ..			14.56	
Theoretical concentration of Av. Cl <sub>2</sub> .. ..			7.68%	
Efficiency per cent .. ..	32.1	33.46	37.63	31.64
Final concentration of Av. Cl <sub>2</sub> .. ..	2.47%	2.57%	2.89%	2.43%
		Added 1% lime		Added 1% lime
<i>Stability.</i>				
Av. Cl <sub>2</sub> after 6 weeks .. ..	2.46%	2.50%	2.83%	2.06%

## EXPERIMENT V.

16-11-1918.

*Saturated solution versus 10 per cent solution.*

	A	B	C	D
Volume per cell .. ..			250 c.c.	
Anode .. ..	Arc Lamp carbon,	6 mm.	diameter	
Cathode .. ..	D i t t o			
Current .. ..	5 amperes			
Brine .. ..	Table salt, saturated,	10 %		
CaO .. ..	1 %	1 %	1 %	1 %
Sodium rosinate .. ..	Nil.	5 c.c.	Nil.	5 c.c.
Any other addition .. ..				
Ampere hours .. ..		12.74		
Final concentration of Av. Cl <sub>2</sub> .. ..	2.57%	2.66%	2.15%	2.07%
Efficiency per cent .. ..	38.24	39.58	32.0	30.8
Theoretical concentration of Av. Cl <sub>2</sub> .. ..			6.72	
	2.80%	2.52%	A & B re-electrolysed until	
<i>Stability.</i>				
Av. Cl <sub>2</sub> after 3 weeks .. ..	2.65%	2.24%	1.59%	1.73%

## EXPERIMENT VI A.

*Experiment showing the efficiency of Electrolysis at 250 c.c. per cell. Table salt solution 25 per cent and 1 per cent lime current 5 amperes.*

Ampere hours	Theoretical Cl. %	Actual Cl. %	Overall Efficiency %	Efficiency of stage %
1:82 ..	0.96	0.8	83	83
3:04 ..	1.92	1.39	72	64.5
5:46 ..	2.88	1.92	67	55.2
7:28 ..	3.84	2.30	59.3	39.5
9:10 ..	4.80	2.46	51	16.6
10:92 ..	5.76	2.58	44.7	12.2
12:74 ..	6.72	2.60	38.5	2.0

*Re-electrolysis after 2 days.*

14:56 ..	7.68	2.74	36	14.6
16:38 ..	8.64	2.82	32.5	8.3
18:20 ..	9.60	2.80	29.0	Negative
20:02 ..	10.56	2.72	24	"
21:84 ..	11.52	2.66	22	"
23:66 ..	12.48	2.73	22	"
25:48 ..	13.44	2.74	20	Nil.

## EXPERIMENT IX.

23-12-1918.

*Effect of Magnesia on stability.*

	B	C	D	E	F
Volume per cell ..	250 c.c.	250 c.c.	255 c.c.	250 c.c.	250 c.c.
Anode ..	Arc	Lamp	carbon, 6	m.m. diameter	
Cathode ..		D i s t a n t			
Current ..		5 a m p e r e s			
Brine ..		Rock salt 25 % in distilled water			
Lime (CaO) ..	1 %	1 %	Nil.	1 %	1 %
Saturated solution sodium ..	Nil.	Nil.	1 c.c.	1 c.c.	1 c.c.
rosinate					
Any other addition ..	Nil.	2.5 % MgO	Nil.	Nil.	2.5 % MgO
Ampere hours ..			16.38		
Theoretical percent chlorine ..			8.64 %		
Av. Cl.					
Efficiency per cent. ..	34.37	33.45	32.41	34.37	34.84
Final concentration of Av. Cl.	2.97 %	2.89 %	2.80 %	2.97 %	3.01 %
(No lime added at the end)					
<i>Stability.</i>					
After 6 weeks ..	2.26	2.23	0.80	2.20	2.62
After 24 weeks ..	0.59	0.79		0.50	0.57

## EXPERIMENT XI.

28-3-1919.

*Platinum Anode and Carbon Cathode versus Carbon Anode and Platinum Cathode.*

	A	B
Volume per cell .. .. .	75 c.c.	75 c.c.
Anode .. .. .	14 mm. gas carbon	Platinum wire
Cathode .. .. .	Platinum wire	Gas carbon
Current .. .. .	5 amperes	
Brine .. .. .	Rock salt 25 %	
Water .. .. .	Distilled	
Lime, CaO .. .. .	1 % after 1.8 ampere hours	
Saturated solution sodium rosinate .. .. .	Nil.	
Any other addition .. .. .	Nil.	
Ampere hours .. .. .	4.91	
Theoretical concentration of Av. Cl <sub>2</sub> .. .. .	8.64	
Efficiency per cent. .. .. .	29.97	39.58
Final concentration of Av. Cl <sub>2</sub> .. .. .	2.59%	3.42%
<i>Stability.</i>	(Added 1 % lime at the end)	
Av. chlorine after 6 weeks .. .. .	2.04 %	2.825%
„ 23 „ .. .. .	Not kept	2.385%

## EXPERIMENT XIII.

1-5-1919.

*Platinum Anode and Carbon Cathode versus Carbon Anode and Platinum Cathode.*

	A	B
Volume per cell .. .. .	250 c.c.	250 c.c.
Anode .. .. .	Platinum wire	14 m.m. gas carbon
Cathode .. .. .	14 m.m. gas carbon	Platinum wire
Current .. .. .	5 amperes	5 amperes
Brine .. .. .	Rock salt 25 %	Rock salt 25 %
Water .. .. .	Distilled	Distilled
Lime, CaO .. .. .	1 %	1 %
Saturated solution of sodium rosinate .. .. .	0.1 %	0.1 %
Any other addition .. .. .	Nil.	Nil.
Ampere hours .. .. .	....	16.38
Theoretical concentration of Av. Cl <sub>2</sub> .. .. .	....	8.64 %
Efficiency per cent. .. .. .	48.26	32.06
Final concentration of Av. Cl <sub>2</sub> .. .. .	4.17 %	2.77 %
<i>Stability.</i>	(Added 1 % lime at the end)	
Av. Cl <sub>2</sub> after 24 hours .. .. .	4.17	2.77
„ 6 weeks .. .. .	3.40 %	2.42 %

## EXPERIMENT XIV.

21-5-1919.

*Effect of adding no lime.*

	A	B
Volume per cell	4 cells of 1000 c.c.	4 cells of 1000 c.c.
Anode	14 mm. gas carbon	14 mm. gas carbon
Cathode	Do.	Do.
Current	6 amperes	6 amperes
Brine	Rock salt 25 %	Rock salt 25 %
Water	Distilled	Distilled
Lime, CaO	<i>Nil.</i>	<i>Nil.</i>
Saturated solution of sodium rosinate	<i>Nil.</i>	<i>Nil.</i>
Any other addition	<i>Nil.</i>	<i>Nil.</i>
Ampere hours	27.3	27.3
Theoretical concentration of Av. Cl <sub>2</sub>	3.6%	3.6%
Efficiency per cent	50.0	50.0
Final concentration of Av. Cl <sub>2</sub>	1.8%	1.8%
	(No lime added)	(No lime added)
<i>Stability</i>		
Av. Cl <sub>2</sub> after 24 hours	0.2% Cl <sub>2</sub> (Added 1 % lime after 24 hours and re-electrolysed)	0.2 % Cl <sub>2</sub> concentration 2.7 %.
	After 48 hours	0.8 %.

## EXPERIMENT XIV A.

7-6-1919.

*Effect of K<sub>2</sub>C<sub>2</sub>O<sub>4</sub> on concentration and stability.*

	A	B	C	D
Volume per cell	..	1200 c.c.	..	..
Anode	Platinum wire	Platinum wire	Platinum foil	Platinum foil
Cathode	Gas	Carbon	14 mm.	14 mm.
Current	6.5	Amperes		
Brine	Sambhar Salt 25%	Rock salt		25 %
Water	Distilled	Distilled	Distilled	Distilled
Lime, CaO		1%		
Saturated solution of sodium rosinate		0.4%		
Any other addition	<i>Nil.</i>	<i>Nil.</i>	8.4 C.C. K <sub>2</sub> C <sub>2</sub> O <sub>4</sub>	<i>Nil.</i>
Ampere hours			27.7	
Theoretical concentration of Av. Cl <sub>2</sub>		3.0 %		
Efficiency per cent	68.8	80.0	85.57	78.03
Final concentration of Av. Cl <sub>2</sub>	2.1%	2.4 %	2.61	2.38%
<i>Stability</i>				
Av. Cl <sub>2</sub> after 6 weeks	0.3	1.15%	2.26%	1.4%

## EXPERIMENT XV.

25-7-1919.

*Effect of Borax on stability in high concentration.*

Volume per cell	..	..	..	2 beakers each 1,000 c.c.
Anode	..	..	..	Platinum wire
Cathode	..	..	..	Acheson graphite
Anode current density	..	..	..	1
Current	..	..	..	6 amperes
Brine	..	..	..	20 % Rock salt, distilled water
CaO	..	..	..	<i>Nil.</i>
Saturated solution of sodium rosinate	..	..	..	<i>Nil.</i>
Any other addition	..	..	..	<i>Nil.</i>
Ampere hours	..	..	..	28.2
Theoretical concentration of Av. Cl <sub>2</sub>	..	..	..	3.72%
Efficiency per cent.	..	..	..	42.5
Final concentration of Av. Cl <sub>2</sub>	..	..	..	1.53%
				Divided into 7 parts of 250 c.c. each and added

	A	B	C	D	E	F	G
	11 grms.	7.5 grms.	3.75 grms.	1% CaO	1% CaO & S.R.	5 c.c. of 1% NaOH	Control
<i>Stability.</i>		Borax					
Av. Cl <sub>2</sub> after 24 hours	1.34%	1.27%	1.22%	1.425%	1.43%	0.37%	0.27%
After 3 weeks	0.42%	0.35%	0.30%	1.38%	1.39%	<i>Nil.</i>	<i>Nil.</i>
"   6   "	..	..	..	1.38%	1.39%	..	..
"   23   "	..	..	..	1.27%	1.27%	..	..

## EXPERIMENT XV A.

29-7-1919.

*Effect of Borax on stability in low concentration.*

Volume per cell	..	..	..	1 cell 1,000 cc.
Anode	..	..	..	Platinum wire
Cathode	..	..	..	Acheson graphite
Anode current density	..	..	..	1
Current	..	..	..	5 amperes
Brine	..	..	..	4% rock salt, distilled water
CaO	..	..	..	Nil.
Saturated solution of sodium rosinate	..	..	..	Nil.
Any other addition	..	..	..	Nil.
Ampere hours	..	..	..	5.1
Theoretical concentration of Av. Cl <sub>2</sub>	..	..	..	0.67%
Efficiency per cent	..	..	..	78
Final concentration of Av. Cl <sub>2</sub>	..	..	..	0.50%

Divided into 4 parts of 250 c.c.  
each and added

	A	B	C	D
	3.75 gms.	2.7 gms.	2.5 cc. CaO	Control No addition
<i>Stability.</i>				
Av. Cl <sub>2</sub> after 24 hours	0.49	0.49	0.51	0.42%
After 3 weeks	0.41%	0.48%	0.51	0.63%
" 7 Months	0.25	0.22	0.43	

## EXPERIMENT XVI.

31-7-1919.

*Optimum amount of lime to be added.*

				A	B	C
Volume per cell .. ..	..	..	..	....	1000 c.c. each	....
Anode .. ..	..	..	..	Platinum wire	Platinum wire	Acheson graphite terminal electrode
Cathode .. ..	..	..	..	....	Gas carbon 14 mm.	....
Current .. ..	..	..	..	....	7 Amperes	....
Brine .. ..	..	..	..	....	20 % rock salt in tap water	....
CaO .. ..	..	..	..	0.5%	Nil.	Nil.
Saturated solution of sodium rosinate ..	..	..	..	0.1%	0.1%	0.1%
Any other addition .. ..	..	..	..	....	Nil.	....
Ampere hours .. ..	..	..	..	....	21.84	....
Theoretical concentration of Av. Cl <sub>2</sub> ..	..	..	..	....	2.88%	....
Efficiency per cent .. ..	..	..	..	82.30	62.50	53.12
Final concentration of Av. Cl <sub>2</sub> ..	..	..	..	2.37%	1.80%	1.53%

B and C mixed and divided into 5 parts of 400 c.c. each and added

		(i)	(ii)	(iii)	(iv)	Control
<i>Stability.</i>		0.25%	0.5% lime	1 %	2 %	Nil.
Av. Cl <sub>2</sub> Initial ..	2.37%	1.54%	1.53 %	1.52%	1.51%	1.46%
After 24 hours ..	2.36%	1.515%	1.53 %	1.52%	1.51%	0.2 %
.. 3 weeks ..	2.28%	1.22 %	1.49 %	1.49%	1.49%	..
.. 6 " ..	2.23%	0.89 %	1.475%	1.49%	1.49%	..
.. 23 " ..	1.99%	Nil.	1.35 %	1.38%	1.37%	..

## EXPERIMENT XVIII.

5-1-1920.

*The effect of adding lime before and after electrolysis.*

				A	B
Volume per cell	..	..	..	1000 c.c.	1000 c.c.
Anode	..	..	..	Platinum wire	Platinum wire
Cathode	..	..	..	Acheson graphite	
Anode current density	..	..	..	1	1
Current	..	..	..	6 Amperes	
Brine	..	..	..	20 % Sambhar salt in distilled water.	
CaO	..	..	..	0.5 %	Nil.
Saturated solution of sodium rosinate	..	..	..	Nil.	Nil.
Any other addition	..	..	..	Nil.	Nil.
Ampere hours	..	..	..	22.20	22.20
Theoretical concentration of Av. $\text{Cl}_2$	..	..	..	2.93 %	2.93 %
Efficiency per cent	..	..	..	76.11	45.95
Final concentration of Av. $\text{Cl}_2$	..	..	..	2.23 %	1.32 %
				A was divided into 2 parts, one kept as it is, to the other added 1 c.c. of Kerosene. (Kept in the cupboard at room temperature). Control: Kerosene.	
<i>Stability.</i>					
Av. $\text{Cl}_2$ after 2 months	..	..	..	2.18 %	1.31 %

## BULK 16.

19-6-1919.

*Stability at different temperatures.*

	A	B	C	D
Volume per cell ..	4 cells	1200 c.c.	....	....
Anode .. ..	Platinum Foil Gas carbon, 14 mm			
Cathode .. ..	14 mm. Gas carbon			
Anode current density ..	1	1	1	1
Current .. ..	..	6 Amperes	....	....
Brine .. ..	25%	Rock salt		
CaO .. ..	1%	1%	1%	1%
Saturated solution of sodium rosinate ..	0.1%	0.1%	0.1%	0.1%
Any other addition ..	0.6 grms.	....	K <sub>2</sub> CrO <sub>4</sub>	....
Ampere hours ..	36.4	36.4	36.4	36.4
Theoretical concentration of Av. Cl <sub>2</sub> .. ..	4.0%	4.0%	4.0%	4.0%
Efficiency per cent ..	73.50	64.50	64.25	55.0
Final concentration of Av. Cl <sub>2</sub> .. ..	2.94	2.58	2.57	2.20%
Mixed A, B, & C. 2.68 %				
Divided into 3 parts and put in white bottles				

	A	B	C	
Room temperature ..	....	22° C	37° C	
Exposed to light ..	....	Incubator	Incubator	
<i>Stability.</i>				
Available Chlorine after 2 months ..	2.15%	2.61%	1.92%	2.0%
„ 8½ „ ..	1.50	2.26	0.2	....

## BULK 16.

*Stability at different temperatures, 20.6-19 to 27.4-20.*

See curve B.

Number of days.			Room temperature on the sheet. 33.5 to 18° C.	22° C. Incubator	27° C. Incubator
Per cent. available chlorine					
Initial	..	..	2.68	2.68	2.68
1	..	..	2.645	2.66	2.63
3	..	..	2.65	2.67	2.63
4	..	..	2.64	2.67	2.60
5	..	..	2.63	2.68	2.55
8	..	..	2.60	2.66	2.50
10	..	..	2.59	2.71	2.49
12	..	..	2.56	2.67	2.43
14	..	..	2.53	2.65	2.39
17	..	..	2.49	2.66	2.34
20	..	..	2.46	2.64	2.28
24	..	..	2.44	2.64	2.21
26	..	..	2.41	2.64	2.17
32	..	..	2.37	2.64	2.10
34	..	..	2.36	2.64	2.09
39	..	..	2.38	2.62	2.04
45	..	..	2.23	2.60	1.91
56	..	..	2.15	2.61	1.91
68	..	..	2.04	2.61	1.78
77	..	..	2.01	2.59	1.73
87	..	..	1.94	2.55	1.625
113	..	..	1.79	2.55	1.40
137	..	..	1.73	2.50	1.42
153	..	..	1.62	2.38	0.96
186	..	..	1.60	2.38	—
241	..	..	1.50	—	—
259	..	..	1.50	2.26	0.99
312	..	..	1.34	2.19	—

## BULK 22.

14-8-1919.

*Comparison of Acheson Graphite and Platinum as Anodes.*

	A	B	C
Volume per cell .. .. .	2000 c.c.	2000 c.c.	2800 c.c.
Anode .. .. .	Platinum wire	Acheson graphite	Platinum wire
Cathode .. .. .	Acheson graphite	14 mm. gas carbon	14 mm. gas carbon
Anode current density .. .. .	1	1	1
Current .. .. .	6 Amperes	6 Amperes	6 Amperes
Brine .. .. .	20 %	Rock salt in tap water	1 %
CaO .. .. .	1 %	1 %	1 %
Saturated solution of sodium rosinate .. .. .	0.1 %	0.1 %	0.1 %
Any other addition .. .. .	Nil.	Nil.	Nil.
Ampere hours .. .. .	55.33	55.33	55.33
Theoretical concentration of Av. Cl <sub>2</sub> .. .. .	3.6 %	3.6 %	2.61 %
Efficiency per cent .. .. .	60.14	62.78	88.12
Final concentration of available chlorine .. .. .	2.165 %	2.26 %	2.30 %

## BULK 23.

23-8-1919.

*Effect of Potassium Chromate.*

	A	B
Volume per cell .. .. .	1250 c.c.	1250 c.c.
Anode .. .. .	Acheson graphite	Acheson graphite
Cathode .. .. .	14mm gas carbon	14mm. gas carbon
Anode current density .. .. .	1	1
Current .. .. .	5 Amperes	5 Amperes
Brine .. .. .	20 %	Rock salt in tap water
CaO .. .. .	1 %	1 %
Saturated solution of sodium rosinate .. .. .	0.1 %	0.1 %
Any other addition .. .. .	Nil.	0.05 % K <sub>2</sub> CrO <sub>4</sub>
Ampere hours .. .. .	2.548	2.548
Theoretical concentration of Av. Cl <sub>2</sub> .. .. .	2.68	2.68
Efficiency per cent .. .. .	67.16	70.90
Final concentration of Av. Cl .. .. .	1.80 %	1.90 %
<i>Stability.</i>		
Available chlorine after 1 month .. .. .	1.66 %	1.86 %
.. 6½ months .. .. .	....	1.48 %

25-1-1919.

*Effect of Carbon and Graphite on Stability.*

Bulk 1, titrating 2.40 per cent available chlorine was used for testing the effect of graphite. About 200 c.c. was put in a blue bottle and about 1 gm. of graphite pure added. Another was kept as control. Titrations were made occasionally. Both were kept in the cupboard.

				Control.	Graphite.
				—	—
27th January	..	..	2.40 %	2.36 %	
28th ..	..	..	2.40 "	2.34 "	
29th ..	..	..	2.40 "	2.34 "	
15th February	..	..	2.29 %	2.22 %	

To compare the effect of carbon on stability, to a portion of the electrolysed brine, some carbon shavings from electrode (gas carbon) were added and kept.

After 24 hours the following were the concentration:

Control	..	..	2.09%Cl <sub>2</sub>	
Carbon	..	..	0.85 %	after 24 hours
Do	..	..	0.5 "	48 "
			0.26 "	3 days

10-9-1919.

*Effect of dilution on Stability.*

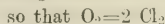
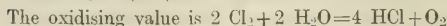
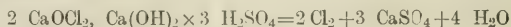
A sample of E. C. 5 days old and titrating 1.86 per cent Cl<sub>2</sub> was divided into 2 parts, one was kept in a green bottle as it is; to the other was added an equal amount of distilled water and kept in a green bottle. The 'dilute' one titrated 0.94 per cent.

				Dilute.	Strong.
				—	—
10th September	..	..	0.94	1.86	
11th ..	..	..	0.91	1.84	
12th ..	..	..	0.925	1.85	
15th ..	..	..	0.92	1.84	
18th ..	..	..	0.91	1.83	
25th ..	16 days	..	0.92	1.79	
5th January	120	..	0.90		
5th March	180	..	0.86		

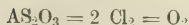
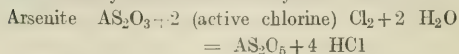
*Note on the term 'Available Chlorine' by Mr. W. A. Davis.*

The term 'available chlorine' is a conventional one which was originally introduced in connection with the bleaching value of bleaching powder and bleaching liquors like Eau de Javelle (potassium or sodium hypochlorite). It is always defined as the weight of chlorine which is *chemically equivalent to the nascent oxygen* which it can furnish (see, for example, Clowes and Coleman, 11th Edn., p. 161).

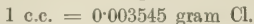
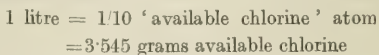
In good bleaching powder containing 40 per cent of available chlorine which is supposed to correspond with the composition  $2 \text{ CaOCl}_2, \text{ Ca(OH)}_2$ , the whole of the chlorine becomes available for oxidising purposes, on treatment with acid.



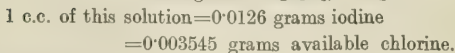
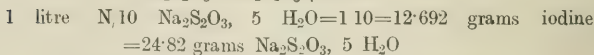
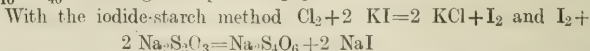
The ordinary solutions for analysis are made on this assumption.



The decinormal solution of arsenite is made so that



Since  $\text{As}_2\text{O}_3 = 4 \text{ Cl}$ , the litre of N.10 arsenite solutions contain

$$\frac{\text{As}_2\text{O}_3}{4 \times 10} = \frac{198}{40} = 4.95 \text{ grams pure } \text{As}_2\text{O}_3$$


In the case of good bleaching powder the available chlorine is the *whole* of the chlorine present. But with sodium hypochlorite solutions we have the oxidising value  $\text{NaOCl} = \text{NaCl} + \text{O}_2$  and the *available chlorine*, which is calculated from the oxidising powder, is twice the actual chlorine present in the molecule, since the oxidising value  $\text{O}_2/2 = \text{Cl}_2$  (from the equation  $\text{Cl}_2 + \text{H}_2\text{O} = \text{HCl} + \text{O}_2/2$ .)

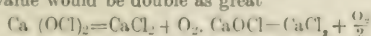
If the available chlorine is determined by the arsenite method or iodide method, 1 c.c. N.10 solution = 0.003545 grams Cl, which is based

on the oxidising value and the amount of available chlorine is twice the actual chlorine present in the  $\text{NaOCl}$ .

What is determined is not the actual chlorine but twice this value which expresses its oxidising power. This is the value which must be used in measuring the disinfection effect. In Metcalf and Eddy's *American Sewage Practice* this is clearly stated (Vol. II, p. 761).

'Each molecule of hypochlorous acid contains one atom of chlorine and one of oxygen and as an atom of oxygen has twice the combining power of the chlorine atom it follows that the oxidising power of the compound is twice as great as its chlorine content . . . . . The words 'available chlorine' simply constitute a term by which is expressed the power of the compound to oxidise or disinfect.'

Metcalf and Eddy's statement 'If a given sample of bleaching powder contains 20 per cent of chlorine in the form of calcium hypochlorite its potency is double this and therefore it is said to contain 40 per cent of available chlorine though in reality it does not, may lead to misinterpretation. Actually the weight of available chlorine in good bleaching powder corresponds with the whole of its chlorine. (See, for example, Cowes and Coleman, p. 161.) This is because in bleaching powder the compound present is not hypochlorite  $\text{Ca}(\text{OCl}_2)$ , but  $\text{CaOCl}_2$ . If it were hypochlorite the oxidising value would be double as great



In connection with electrolytic liquors consult Lunge Keane 'Technical Methods of Chemical Analysis,' Vol. I, Part 1, p. 509, et seq. The presence of free hypochlorous acid has to be taken into account on which the exceptionally great bleaching value of electrolytic liquors depends. Note the statement that 'insoluble substances such as zinc oxide, *Calcium Hydroxide* may be taken as without action on hypochlorous acid.' This suggests that suspended slaked lime neutralises hypochlorous acid very slowly. The methods of analysis for free hypochlorous acid are given. The reference given to James and Richey, J. Amer. Chem. Soc. 1902, 24, 469, for methods of analysis in electrolytic works may be useful.

Copy of a letter No. 3349, dated the 14th May 1919, from the Director, Bombay Bacteriological Laboratory, Parel, Bombay, to the Assistant Director-General, Indian Medical Service (Stores), Simla.

With reference to your No. 96-1139-1-19 of February, 1919, and in continuation of yesterday's telegram *re* Hutchinson's Hypochlorite

solution, I have the honour to give in the following table the results of weekly examination :—

Date of estimation of the available chlorine.	PERCENTAGE OF AVAILABLE CHLORINE IN THE BOTTLE, KEPT	
	In the light.	In the dark.
27-3-19 .. .. .	2.234	2.284
3-4-19 .. .. .	2.205	2.279
10-4-19 .. .. .	2.179	2.272
17-4-19 .. .. .	2.165	2.264
24-4-19 .. .. .	2.123	2.251
1-5-19 .. .. .	2.045	2.250
8-5-19 .. .. .	1.973	2.194

From the above results it will be seen that in six weeks' time the sample exposed to light has deteriorated in its available chlorine content by 11.7 per cent, while that kept in the dark by a little less than 4 per cent.

Bacteriological examination was not done, as the efficiency of such preparations depends solely on the quantity of available chlorine contained therein.

I have had some experiments made with the electrolytic chlorogen you supplied, of which I should give you the results. In regard to efficiency, you will see from the tables in the file, that it readily eliminated pathogenic organisms added to tap water as an artificial contamination, but that it does not eliminate spore bearing organisms in a naturally contaminated water. There is, of course, nothing new in these observations, which were carried out for my own edification, but it may interest you to know that the loss of available chlorine in 3 months and 6 days was only 0.18 per cent from a bottle left on the bench, exposed to daylight, and to frequent handling. I have made no observations on its behaviour at plains temperature.

(Sd.) T. C. McCOMBIE YOUNG.

11th September, 1919.

*Sanitary Commissioner, Assam.*

EXPERIMENT 1. 17TH AUGUST, 1918.

*Sample No. 10. River water sample.*

1. *Control.*

1 c.c. of river water plated on agar ; incubated overnight at 37 °C ; colonies innumerable.

2. 1 in 20,000 dilution *E. C.* No. 10.

39 c.c. tap water plus 1 c.c. *E. C.* No. 10 equals 1 in 40 dilution

1 c.c. of 1 in 40 dilution plus 499 c.c. of river water : equals 1 in 20,000 dilution.

Contact half hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : no colonies.

3. 1 in 40,000 dilution *E. C.* No. 10.

5 c.c. of above 1 in 40 dilution plus 5 c.c. tap water equals 1 in 80 dilution.

1 c.c. of 1 in 80 dilution plus 499 c.c. river water ; equals 1 in 40,000 dilution.

Contact half hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : no colonies.

EXPERIMENT 2. 17TH AUGUST, 1918.

*E. C. sample No. 33. Same river water sample.*

1. *Dilution 1 in 20,000 E. C. No. 33.*

39 c.c. tap water plus 1 c.c. *E. C.* No. 33 equals 1 in 40 dilution.

1 c.c. of 1 in 40 plus 499 c.c. of river water sample, equals 1 in 20,000 dilution.

Contact half hour at room temperature.

1 c.c. plated on agar. Incubated overnight at 37°C.

Result : no colonies.

2. *E. C. No. 33. Dilution 1 in 40,000.*

5 c.c. of above 1 in 40 dilution plus 5 c.c. tap water, equals 1 in 80 dilution.

1 c.c. of 1 in 80 dilution plus 499 c.c. of river water, equals 1 in 40,000 dilution.

Contact half hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : no colonies.

3. *Dilution 1 in 40,000 E. C. No. 33.*

5 c.c. of above 1 in 40 dilution plus 5 c.c. tap water ; equals 1 in 80 dilution.

1 c.c. of 1 in 80 dilution plus 499 c.c. of above B. coli emulsion ; equals 1 in 40,000 dilution.

Contact half hour at room temperature

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : no colonies.

## EXPERIMENT 3. 22ND AUGUST, 1918.

*Water from river Um Susum in which a laboratory culture of B. coli has been emulsified.*

E. C. sample No. 10.

1. *Control.*

1 c.c. of B. coli emulsion plated on agar ; incubated overnight at 37° C.

Result : colonies innumerable.

2. *Dilution 1 in 20,000. E. C. No. 10.*

1 c.c. of E. C. No. 10 plus 39 c.c. tap water ; equals 1 in 40 dilution.

1 c.c. of 1 in 40 dilution plus 499 c.c. of the above B. coli emulsion ; equals 1 in 20,000 dilution.

Contact half an hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37°C.

Result : 4 colonies. (4)

3. *Dilution 1 in 40,000. E. C. No. 10.*

5 c.c. of above 1 in 40 dilution plus 5 c.c. tap water ; equals 1 in 80 dilution.

1 c.c. of 1 in 80 dilution plus 499 c.c. of above B. coli emulsion ; equals 1 in 40,000 dilution.

Contact half an hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : 34 colonies.

## EXPERIMENT 4. 22ND AUGUST 1918.

*E. C. sample No. 33. Same B. coli emulsion as above, in river water.*

1. *Dilution 1 in 20,000 E. C. No. 33.*

1 c.c. of E. C. No. 33 plus 39 c.c. tap water ; equals dilution 1 in 40.

1 c.c. of 1 in 40 dilution plus 499 c.c. of above B. coli emulsion ;  
equals 1 in 20,000 dilution.

Contact half an hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37° C.

Result : no colonies.

2. *Dilution* 1 in 40,000. *E. C. No. 33.*

5 c.c. of above 1 in 40 dilution plus 5 c.c. tap water ; equals  
1 in 80 dilution.

1 c.c. of 1 in 80 dilution plus 499 c.c. of above B. coli  
emulsion ; equals 1 in 40,000 dilution.

Contact half an hour at room temperature.

1 c.c. plated on agar ; incubated overnight at 37°C.

Result : no colonies.

THE RELIABILITY OF GATE AND  
PAPACOSTAS' FORMOL-GEL TEST  
FOR SYPHILIS AS COMPARED  
WITH THE WASSERMANN  
REACTION.

BY

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*Civil Assistant Surgeon attached to the King Institute, Madras.*

*(From the King Institute of Preventive Medicine, Guindy, Madras.)*

[Received for publication, June 11, 1921.]

In the Comptes Rendus des Seances de la Société de Biologie for November 20th, 1920 (an abstract<sup>2</sup> of which appeared in the *British Medical Journal* for January 15th, 1921) Gaté and Papacostas<sup>1</sup> describe a new test for syphilis which, if proved to be reliable under all conditions, would be of very considerable value in the diagnosis of the disease on account of its extreme simplicity and the rapidity with which it can be performed.

The test consists in the addition of a small quantity of formalin to the suspected serum. Two drops of the reagent are added to 1 c.c. of the serum and, after shaking, the mixture is left for 24 to 30 hours. The results are then read. A positive reaction is denoted by the formation of a more or less solid gel. A serum which is negative remains fluid. The authors claim that the test has given them 85 per cent of agreement when compared with the Wassermann reaction and that, in those cases in which the two tests disagreed, they were uncertain which result was the more accurate.

In the face of these results it appeared desirable that the test should be further investigated with a view to ascertaining whether such a high percentage of agreement could be corroborated and whether the cases of disagreement could be limited to any particular type of the disease as defined by the Wassermann reaction.

With this end in view the blood of all cases which have been sent to this laboratory for a diagnosis of syphilis have been tested by both methods. No selection as to any particular type of case has been made. The only criterion by which the suitability of each case has been gauged has been the sufficiency of serum for both tests. In this way 539 sera from all sources have been examined. The particular modification of the Wassermann reaction which has been employed is that described by the Medical Research Council (with one or two minor modifications) in which varying doses of complement are titrated with a fixed quantity of amboceptor. This method has now been in use at the King Institute for over two years and has always given consistent results.

In a considerable number of cases the requisite quantity of serum laid down by the authors (1 c.c.) was not available after the amount required for the Wassermann test had been deducted. The cases have, therefore, been divided into two series according to the treatment they have received. In the first, the exact proportion of formalin to serum given by the authors has been adhered to *i.e.*, a 1 in 8.5 dilution, taking 1 drop as the equivalent of 1 minim and 1 c.c. as 17 minims) and the reagents have been measured by volume by means of a Wright's pipette. In the second series the measurement has not been quite so accurate. The serum has been measured and 1 or 2 drops of formalin have been added according as the quantity of serum approached 0.5 or 1 c.c.

The results obtained with the two series of cases are given in Tables I and II and the combination of the two in Table III.

TABLE I

*Showing a comparison between the results obtained with the Wassermann and formol-gel tests in the first series of cases.*

WASSERMANN REACTION.			FORMOL-GEL REACTION		PERCENTAGE OF AGREEMENT.
	M.H.D. of Complement.	No. of cases.	NO. OF CASES IN		
			Agreement	Disagreement.	
POSITIVE, ..	8	36	24	12	66·6
	5	27	18	9	66·6
	3	24	11	13	45·8
TOTAL ..	..	87	53	34	61·1
NEGATIVE ..	..	113	68	45	60·1
TOTAL ..	..	200	121	79	60·7

TABLE II

*Showing a comparison between the results obtained with the Wassermann and formol-gel tests in the second series of cases.*

WASSERMANN REACTION.			FORMOL-GEL REACTION		PERCENTAGE OF AGREEMENT.
			NO. OF CASES IN		
	M.H.D. of Complement.	No. of cases.	Agreement.	Disagreement	
POSITIVE ..	8	58	45	13	77
	5	49	35	14	71·5
	3	43	30	13	70
TOTAL ..	..	150	110	40	73·3
NEGATIVE ..	..	189	129	60	68
TOTAL ..	..	339	239	100	70·5

TABLE III

*Showing a comparison between the results obtained with the Wassermann and formol-gel tests in two series of cases combined.*

WASSERMANN REACTION.			FORMOL-GEL REACTION.		PERCENTAGE OF AGREEMENT.
			NO. OF CASES IN		
	M.H.D. of Complement.	No. of cases.	Agreement.	Disagreement.	
POSITIVE	8	94	69	25	73.4
	5	76	53	23	69.8
	3	67	41	26	61.2
TOTAL	..	237	163	74	68.8
NEGATIVE	....	302	197	105	65.2
TOTAL	....	539	360	179	66.6

The sera have been divided according as they gave a positive or negative Wassermann reaction and, in the former case, according to the number of minimum hæmolytic doses of complement they were capable of deviating. The results of the formol-gel reaction are stated according as they have been found to be in agreement or disagreement with those of the Wassermann reaction and a percentage of agreement has been struck in each case.

It will be seen that the test has not given the good results claimed for it by its authors in the present series of cases. The percentage of agreement in the first series is only 60.7 and in the second 70.5, giving an average of 65.6. Further, even under the most favourable circumstances when the serum has deviated the highest number of minimum hæmolytic doses and where presumably the syphilitic ambocceptor is present in quantity, the formol gel test has not succeeded in showing more than 73.4 per cent of cases detected by complement deviation.

Under these circumstances the test cannot be considered sufficient accurate to be used as a reliable substitute for the more complicated Wassermann reaction, for a test which misses 30 per cent of marked cases.

## 624 *Reliability of Gaté and Papacostas' Formol-Gel Test.*

cannot compete, even on the grounds of simplicity, with one that gives over 90 per cent of success in cases of the same type.

In the less pronounced reactions the results are still more divergent. In this case, however, the infallibility of the Wassermann test cannot be claimed with nearly so much certainty, and reference to other factors, such as the clinical evidence, would be advisable before a satisfactory opinion on the matter could be given. This further investigation has not been considered necessary, however, in view of the comparatively high rate of discrepancy already noted between the two reactions in pronounced cases of the disease.

### CONCLUSIONS.

Gaté and Papacostas' formol-gel reaction has been tested with the sera of 539 cases sent for the diagnosis of syphilis against the Wassermann reaction and the two tests have been found to agree in only 66·6 per cent of cases.

In the sera which have deviated 8 M.H.D. of complement and which presumably came from well marked cases of the disease the percentage of agreement was only 73·4 per cent.

The test cannot therefore be considered sufficiently reliable to take the place of the Wassermann reaction.

I express my grateful thanks to Major J. Cunningham, M.D., I.M.S., Director of the King Institute, for his valuable help during the progress of these tests.

### REFERENCE.

<sup>1</sup>GATÉ and PAPACOSTAS .. Comptes Rendus Société Biologie, November 20th, 1920.

<sup>2</sup>(Abstract, B. M. J., January 15, 1921.)

# THE HEART IN BERIBERI AND THE EVIDENCE OF THE DIFFERENTIAL STETHOSCOPE.

BY

C. A. SPRAWSON.

[Received for publication, August 9, 1921.]

IN the Mesopotamian Expeditionary Force were several thousand Chinese artificers and labourers, some hundreds of whom were attacked with beriberi in the years 1917 to 1919. An account of this disease in Mesopotamia amongst Chinese, British and Indians has been given by the writer in another paper.<sup>1</sup>

It suffices here to say that many of the Chinese in Mesopotamia appeared to be suffering from 'latent beriberi,' and they were liable to show the well-known cardio-neurotoxic syndrome as a result of any immediate depressing influence. This exciting cause was usually fatigue, over long hours of work, or concurrent illness, such as malaria or influenza.

The predisposing cause of their abnormal condition was perhaps long continued dietetic deficiency in their own country or a previous attack of the disease.

Amongst the hundreds of Chinese beriberi cases the cardiac signs were more carefully investigated in 62 and this brief paper is on the results of this investigation.

For instance, amongst these 62 cases in 27 the *knee jerks* were present, in 18 they were notably increased, and in 17 they were absent. In this connection it may be said that examination of a supposedly healthy body of Chinese labourers will show that some are without the knee-jerk, evidence presumably of the abnormal 'latent beriberi' condition in which many Chinese of this class habitually live.

Eight of the cases showed no *anaesthesia* of the legs or elsewhere ; in 2 the determination was doubtful : in 42 anaesthesia usually, patchy, of the legs, was present : and in 8 cases the anaesthesia was a prominent feature.

Forty-two cases showed no deep *tenderness* on pressure of the calf or thigh muscles, in 6 the determination was doubtful, and in 14 the sign was present.

Fourteen cases had no *oedema*, in 2 its presence in slight amount was doubtful, in 18 there was definite oedema and in 8 cases the oedema was a prominent feature of the disease. The oedema when present in moderate degree was over the shins, especially in their lower part. The feet were usually affected later.

More attention will now be paid to the *circulatory* signs, although it was not for those the patient as a rule sought admission. He nearly always complained of weakness of the legs, only occasionally of shortness of breath.

The *pulse rate* of the 62 cases, taken with the patient lying down, varied from 52 to 126 per minute, the average being 84 on admission. Amongst those who were able to stand the pulse rate on standing varied from 80 to 132, the average being 107.

Thirty-two of the 62 patients were noted as showing abnormal cardiac signs. At the time of admission these were noted as follows :—

Dilatation of heart and	{	Systolic murmur at aortic area	..	..	..	1
		" " " mitral "	..	..	..	4
		" " " pulmonary "	..	..	..	1
		" " " tricuspid "	..	..	..	5
		" " " mitral and tricuspid area	..	..	..	2
		" " " over whole cardiac "	..	..	..	1
Dilatation of heart		..	..	..	..	8
Diffuse impulse		..	..	..	..	2
Accentuation of pulmonary second sound		..	..	..	..	2
Prolongation of first sound		..	..	..	..	1
Very forcible beat		..	..	..	..	1
Tachycardia without other sign		..	..	..	..	4

It appears that the commonest abnormality is dilatation of the heart, usually more pronounced on the right side, and sometimes accompanied by signs of tricuspid regurgitation. The evidence of dilatation was obtained by percussion and by noting the position of the apex beat. The cases were not viewed through the screen.

The dilatation, however, was by no means always right-sided only or more evident on that side. Some of these cases, had they occurred in British or Indian troops, would certainly have been returned under that too comprehensive heading 'Disorderly action of the Heart.' The fact that the men were Chinese rendered further examination advisable resulting usually in the detection of some other feature of the beriberi syndrome. Even if no other feature of beriberi were discoverable it is probable that cardiac dilatation and tachycardia in a Chinese labourer would be of beriberi nature.

Myocardial involvement in beriberi has long been recognised and it is now generally considered that heart failure in this disease is due to direct affection of the heart muscle much more than to any degenerative process in the vagus nerves.

Herzog<sup>2</sup> writes in Osler and Macrae's System of Medicine: 'The myocardium, as a whole, is hypertrophied; this is usually most marked in the right ventricle, but the left may likewise be enlarged. The right ventricle in particular is generally not only hypertrophied, but also markedly dilated, so that a relative insufficiency of the tricuspid valve is present.....The myocardium may be normal, but quite frequently it is found to be more or less cloudy and mottled in consequence of diffuse fatty degeneration.'

Yamagawa<sup>3</sup> mentions first amongst the most important method changes 'Dilatation and hypertrophy of the right ventricle and dilatation of the left; fatty metamorphosis of the myocardium.'

It would appear, therefore, that cases of beriberi are peculiarly suitable for examination by the differential stethoscope and from two points of view: one is to test the value of the information that this stethoscope can give us and the other, if we find the information given trustworthy, to make it a basis of prognosis and occasionally of treatment.

Leyton's stethoscope possesses a sound-box with a needle-valve the screwing down of which lessens the aperture through which the sound may pass, in the same way as the needle-valve in a carburettor may lessen the intake of petrol. The valve is connected to a graduated dial so that the relative area and therefore the relative amount of sound allowed by the various positions of the needle may be read. It is claimed that the intensity of the first heart sound at the apex should be in a healthy person double that of the second heart sound at the aortic area. A lessening of that ratio indicates myocardial weakness.

if the ratio be less than 1·5 to 1 the interpretation is said to be grave myocardial debility. That this is so can be verified by examining a patient towards the end of an attack of enteric fever or similar disease when the myocardium is obviously weak. It is another question however whether this differential stethoscope can give information that we cannot obtain otherwise, by direct observation and by the ordinary stethoscope.

Of the above 62 cases, 55 were examined by Leyton's differential stethoscope. The remaining 7 were unsuitable for such examination; because they showed cardiac murmurs. Seven others it is true also showed murmurs on admission, but in those the murmurs disappeared shortly under treatment by rest in bed and so permitted of their examination.

These 55 patients were examined therefore with the differential stethoscope and the readings compared with those obtained from 11 apparently healthy Chinese of the same class.

Amongst the 55 patients the average ratio of the first sound at apex to second sound at base was 1·6 to 1·0. In 36 cases the ratio was under the normal 2 to 1; in 17 cases the ratio was under 1·5 to 1.

Amongst the eleven apparently healthy Chinese a difficulty arises about one case. This man, a hospital cook, gave an apex reading of 2·6 and a base reading of 3·0. The ratio therefore in this case was less than 1 to 1, and the man should have suffered from grave myocardial debility. He was, however, apparently healthy and complained of nothing: his subsequent history is not known. If this case be included amongst the healthy the average ratio of the eleven was 1·9 to 1. If the case of the cook be excluded the average ratio of the remaining ten was 2·2 to 1·0. Excluding the cook only one other case showed a ratio of under 2 to 1 and none a ratio of under 1·5 to 1.

The deduction therefore is that the beriberi cases as a class, whether showing cardiac signs or not, do exhibit evidence of myocardial degeneration by the differential stethoscope, the ratio of the beriberi cases being 1·6 and of the healthy about 2·0.

The question whether the differential stethoscope can aid us in the prognosis of any individual case of beriberi is more difficult. One must leave statistics here and rely on one's personal clinical impression. An estimate of the number of days spent in hospital by each patient taken into relation with his differential stethoscope ratio would be of no avail for two reasons: firstly, because, after recovery from cardiac

signs the patient may remain in hospital for neurotoxic signs, and secondly because it was the correct policy to evacuate these beriberi cases from the expeditionary force as soon as possible, so that the complete history of many was unknown to the physician in Mesopotamia.

The writer's impression is that the differential stethoscope was of some aid in estimating the amount of myocardial debility in these cases and in forming a prognosis.

Usually one could arrive at a similar conclusion from other data ; but in some cases the additional evidence afforded by the differential stethoscope gave one further confidence in one's conclusions. Further as regards treatment the conclusion arrived at in some cases prompted the medical officer to keep the patient longer in bed than might otherwise have been the case. Since all beriberi cases were evacuated, the differential stethoscope cannot be said to have exercised any influence on the ultimate disposal of cases.

#### CONCLUSIONS.

(1) About half the number of these Chinese beriberi cases showed on admission some cardiac abnormality, which was usually slight enough to disappear shortly after treatment by rest in bed.

(2) There was distinct evidence of myocardial debility, as shown by the differential stethoscope in beriberi cases.

Both sides of the heart may be affected, the right usually somewhat more than the left.

(3) The use of the differential stethoscope was sometimes of aid in prognosis and treatment.

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|-----------------|----|---------------------------------------------------------------------------------------|
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| (2) HERZOG ..   | .. | Article on beriberi in Vol. III of Osler and Macrae's System of Medicine.             |
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## A NOTE ON GRAPPLING TAIL-HOOKS IN ANOPHELINE LARVÆ.

BY

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[Received for publication, August 2, 1921.]

In a paper read before the Indian Science Congress, January 1920, I made a reference to the occurrence and utility of grappling tail-hooks in anopheline larvæ including those of *A. stephensi*, *hyrcanus* (*sinensis*) and *fuliginosus*.\* Prior to this there has been no reference made to the presence of hooks in the setæ on the ninth segment of the abdomen. In the figures drawn by various authors, the supra-anal setæ are represented as straight and pointed at the tips. The object of the present note is to describe, in detail, my observations on the occurrence and utility of these hooks, in a large number of Indian species.

The posterior dorsal region of the anal segment has a set of four tufts of setæ, a median pair and an outer pair (Plate LXI, fig. 1). They start from plates of chitin arranged as in Plate LXI, fig. 2, on the posterior dorsal tip of the anal segment. The median pair starts from either end of a transversely placed strip of chitin, and is anterior and dorsal to the outer pair. The outer tufts start from two curved beak-like plates of chitin on either side of the median line, and which in some cases (*A. hyrcanus*) may be fused at the base (Plate LXI, fig. 3). These plates could only be seen well when the setæ had been pulled out. If the setæ are intact, we cannot get a superficial view of these plates.

The median tufts are of the feathered type and when the larva is at rest, they are projected backwards and upwards from the tip of the anal segment. The branches are in one plane and the tuft is vertically

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\* Preliminary Report of a Malaria Survey of Calcutta, *Indian Journal of Medical Research*, Special Indian Science Congress Number, 1920, pp. 15-16.

# EXPLANATION OF PLATE LXI

Fig. 1. Dorsal views of the ninth segment of the abdomen of anopheline larvae showing M—median tufts, E—external tufts and A G—anal gills.

Figs. 2 & 3. Dorsal tip of the anal segment showing chitin plates supporting the dorsal tufts of *A. pinesti* and *hypogaeus*. (The tufts have been pulled out.) M—the socket of the median tuft E—the socket of the external tuft. Drawn with the Camera lucida, Zeiss ocul. 2, objective AA, tube length 160 mm.

Fig. 4. Lateral view of the posterior region of the abdomen of *A. hypogaeus* larva. Camera lucida drawing, Zeiss oc. 2 obj. AA, T. L. 160 mm.

M. medial supra-anal tuft.

E. external supra-anal tuft showing hooks.

A G. anal gills.

# EXPLANATION OF PLATE LXI.

Fig. 1. Dorsal views of the ninth segment of the abdomen of anopheline larvae showing M—median tuft, E—external tuft and A. G.—

anal gills.

Figs. 2 & 3. Dorsal tip of the anal segment showing chitin plates supporting the dorsal tuft of A. Jawless and Wawana. (The tufts have been pulled out). M—the socket of the median tuft, E—the socket of the external tuft. Drawn with the camera-lucida. Weiss ocular 2, objective AA, tube length

160 mm.

Fig. 4. Lateral view of the posterior region of the abdomen of A. Wawana larva. (camera-lucida drawing Weiss oc. 2 obj. AA, T. R.

160 mm.

M. median supra-anal tuft.

E. external supra-anal tuft showing hooks

A. G. anal gills.

PLATE LXI.

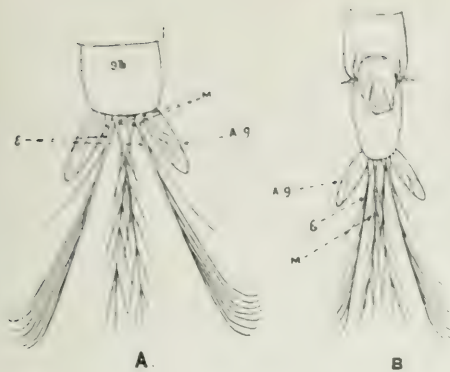


Fig. 1

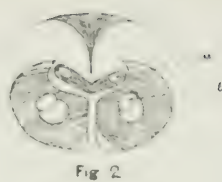


Fig. 2

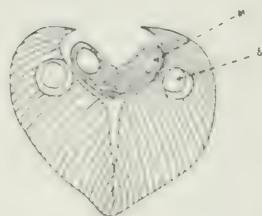


Fig. 3.

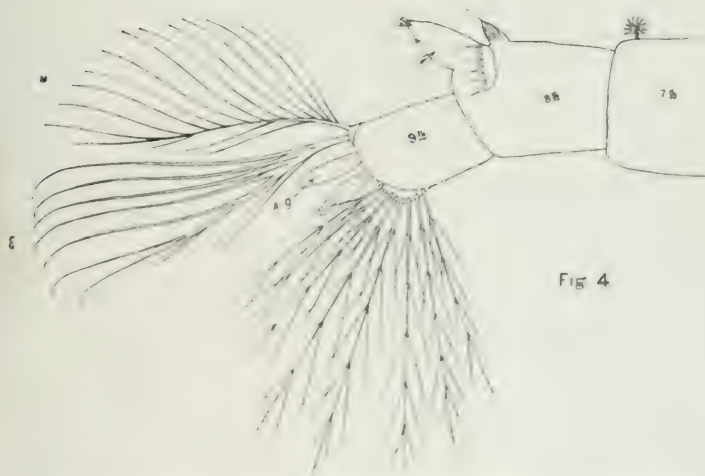


Fig 4





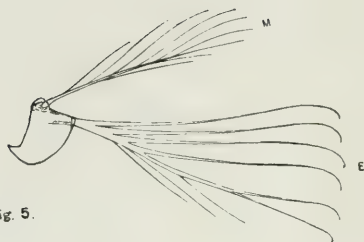


Fig. 5.

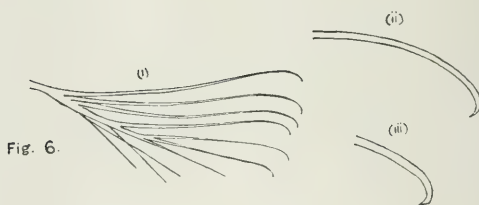


Fig. 6.

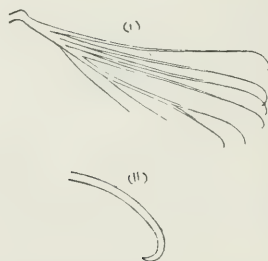


Fig. 7

# EXPLANATION OF PLATE LXII.

Fig. 5. Median and external tufts of one side of the jaws of *A. jamaica*.  
 Camera-lucida, ocular 2, objective *11*.  
 Figs. 6 & 7. External hooked tufts of *A. bahianensis* and jawes and the  
 tips of the hooked hairs magnified; drawn with the Camera-  
 lucida, (i) oc. 2, obj. *11*, (ii) and (iii) oc. 2 and obj. *11*.

EXPLANATION OF PLATE LXII.

Fig 5. Median and external tufts of one side of the larva of *A. jamesi*.  
Camera-lucida, ocular 2, objective AA.

Figs. 6 & 7. External hooked tufts of *A. barbirostris* and *jamesi* and the  
tips of the hooked hairs magnified: drawn with the Camera-  
lucida, (i) oc. 2, obj. AA., (ii) and (iii) oc. 2 and obj. DD.

placed. Frequently, the two tufts may cross each other (Plate LXI, fig. 1, A). It will be seen from Plate LXI, fig. 4, that there are many more branches on the dorsal than on the ventral side. In *A. hyrcanus* there are 12 dorsal and only 4 ventral branches, and in some cases the ventral branches are still fewer.

The outer tufts consist each of about six equally long strongly chitinised setæ the tips of which are bent and sharply hooked and a few (2 to 4 or more) thin branches ventrally which possess no hooks. In some forms, the number of such hooked setæ may be as many as 7-10. The branches are somewhat flat at their bases. The hooks on the external supra-anal setæ are strong and thick. But the branches of the median supra-anal tufts have also been found in a few cases to have very minute hooks at their tips, visible only under the high power. They are very small and feeble, and may not be of any great use. I have found them in the larvæ of *A. maculipalpis*, *culicifacies* and *minimus*.

The following are observations of larvæ of *Anopheles stephensi* breeding in cisterns in Calcutta. When the larva rests along the surface of the water, the hooked setæ are projected backwards and the hooks point downwards (Plates, LXI and LXII, figs. 4-5). When disturbed, the larva goes to the bottom if the water is shallow. But if deeper, it has been observed to go only to the side of the cistern and sink slowly with the tail end touching the side. While thus descending, the hooks catch even the smallest prominence or roughness in the side and the larva hangs head downwards from the wall of the cistern by means of these hooks. It sticks to this position as long as the disturbance continues, and when everything is quiet again comes up to the surface. If from one such resting position the larva is disturbed by touching it gently with a long needle, it either comes up to the surface, or more frequently goes to another place on the cistern side and hangs from the new position in the same way. This led me to suspect the presence of some grappling organs in the tail, and an examination showed that these were the hooks of the external supra-anal tufts. Larvæ can stick to the sides of even a glass-jar in which they may be kept and dangle head downwards for varying lengths of time. In one instance, a larva clung to the sides of a cistern, under water for as long as 3 minutes.

All the species of *Anopheles* available for study have been examined since, including *A. subpictus*, Grassi (*grossi*, Giles); *vagus* Donatz; *culicifacies*, Giles; *fuliginosus*, Giles; *jansei*, Theob; *maculipalpis*,

Giles; *maculatus*, Theob.; *minimus*, Theob.; *funestus* var. *listoni*, Liston; *stephensi*, Liston; *barbirostris*, v. d. Wulp. *hyrcanus*, Pallas and *gigas*, Giles. In all these species, the hooks are present quite characteristically.

In a pond, it may be presumed that if the larva were to be disturbed it would be compelled to go to the bottom. But this has never been observed to happen. The larvæ of Anophelines breeding in ponds with a floating vegetation of *Pistia stratiotes* for instance, get in between the roots of the *Pistia* when disturbed and hang from the rootlets by their tail-hooks. But sometimes they grip the rootlets with their mandibles. From such positions it is difficult to dislodge them. If the larvæ should go to the bottom of the pond on any disturbance, the possible dangers they would risk thereby would be (1) the exposure to enemies like fish and (2) exposure to pressure at the depths of a pond which cannot be withstood by these air-breathing organisms. This also enlightens us as to why larvæ always rest near floating aquatic plants or algal scums, or near the edge of the pond or cistern, and never at the clear centre of a pond.

Above we have seen the utility of the tail-hooks in stagnant waters.\* In flowing waters, the larvæ of species breeding therein stick to boulders on the sides of the streams by means of their well-developed tail-hooks. The larvæ of *A. maculatus* and *minimus* breeding in the 'jhoras' (hill-streams at the foot of the Himalayas) have been observed to do so. But the power to stick to the sides of streams is particularly remarkable with larvæ of *A. maculatus*, and they are able to withstand very strong currents. Experiments in the Bengal Duars have shown that flushing a stream has no great effect in driving the larvæ of *A. maculatus* far down the stream. When the stream gets a good flush, the *maculatus* larvæ breeding in it at once get to the surface of the flush water, move a short distance with the current and suddenly dart out to the sides and there stick to the stones or boulders, in spite of the strong current. When the flush spends itself and the level of the water falls, the larvæ creep down the sides, and when the water is stationary again, get into the water. The larvæ of *A. culicifacies* breeding in the sluggish streams in the colliery districts also utilise their tail-hooks to stick to stones and sand,

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\* It has been said in the paper referred to in the postscript that the floating Anopheles larva sticks to the edge of a glass vessel and maintains itself at right angles to the side of the vessel by means of the two lateral hook tufts. I have observed larvæ in which the tufts were completely removed without injury to the larva and they behave exactly similarly so that, apparently there is no connection between these hooks and the resting position at right angles to the sides of a vessel.

but they have no capacity to withstand a flush. In one instance, a good flush was able to drive all larvæ of *culicifacies* a mile down the stream.

Specimens of larvæ of *A. maculipennis*, Meigen, *bifurcatus*, Linn. and *plumbeus*, Haliday, received through the courtesy of Prof. G. H. F. Nuttall, were also examined. The hooks were quite typical in the two former, but in *A. plumbeus*, the hooks were very few and minute.

I have (through the courtesy of the Director, Zoological Survey of India) seen type specimens of larvæ of *Anopheles annandalei*, Prashad, a tree-hole breeding species from the Himalayas. I have observed that the hooks are present quite characteristically in this species also.

*Postscript*.—Just prior to sending this note for publication, I have seen a paper by W. A. Lamborn on the 'Nature and function of caudal tufts in malayan anopheline larvæ,' in the latest issue of the Bulletin of Entomological Research (Vol. XII, pt. 1, July 1921).

I wish to note briefly on some points raised by Mr. Lamborn. He believes that in previous literature, 'no structural differentiation of the two sets of (dorsal) brushes had been noted' (p. 92). It should be pointed out that Nuttall and Shipley in their articles on the 'Structure and Biology of *Anopheles maculipennis*' (*Journal of Hygiene*, Vol. I, pt. 1, 1901) have clearly described and figured these tufts. Their figures and descriptions are perfect, though they missed these hooks.

Mr. Lamborn says 'The presence of any hooks at all in the case of *A. subpictus* var. *vagus* and others which breed by choice in the still waters of muddy pools is doubtless to be explained by recent modifications of breeding habits: for until the advent of the white man to this country and the subsequent great economic development there must have been comparatively few such breeding places available.' It is here assumed that these hooks are quite useless in stagnant waters; this statement is fully controverted by my note. It is too much to assume that there were few stagnant waters before the advent of the white man; and there is no evidence to show that there has been any 'recent modification of breeding habits.'

Mr. Lamborn notes that tree-hole breeding species have imperfectly developed hooks. I have not seen *A. asiatica*. *A. plumbeus* larvæ from England show this reduction, but the hooks are present though few and feeble. But as stated above the larvæ of *A. annandalei* Prashad, also a tree-hole breeder, show no such reduction the hooks being quite well developed. There is no evidence about the other tree-hole breeding species.

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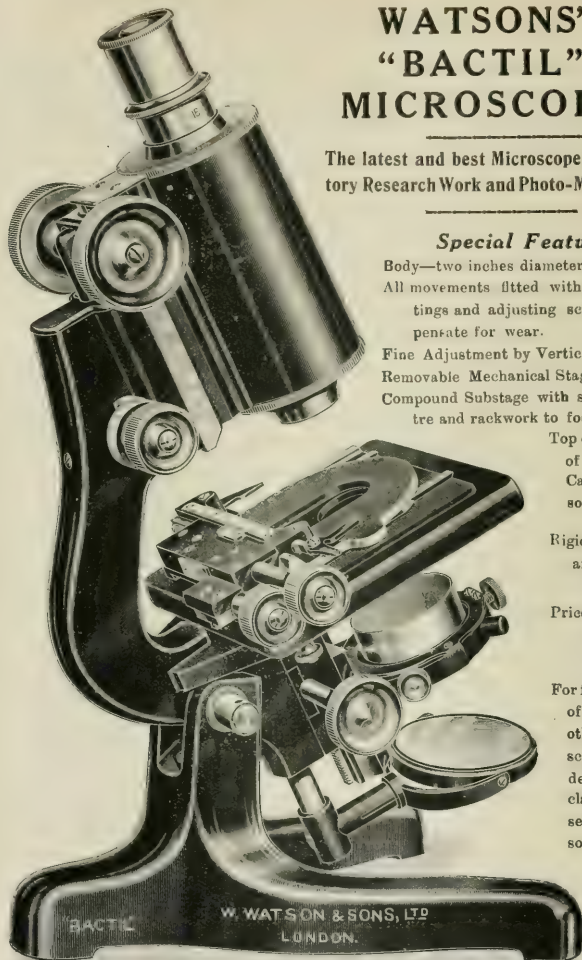
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## KALA-AZAR INQUIRY OF THE INDIAN RESEARCH FUND ASSOCIATION.

### INTIMATION.

In the January 1922 Number of the Journal a telegram from Mrs. Adie was published in which she stated that she had found Leishman-Donovan bodies in the salivary glands and ducts of *Cimex Rotundatus* caught on the bed of a suspected case of Kala-Azar in an infected area. Mrs. Adie's specimens were sent to Lt.-Col. Christophers c.i.e., i.m.s., of the Central Research Institute, Kasauli, for examination, and he has submitted a note on the bodies found by Mrs. Adie in the salivary glands of *Cimex Rotundatus*. Lt.-Col. Christophers considers that the bodies described by Mrs. Adie in the salivary glands of the bed-bug as Leishman-Donovan bodies to be a species of *Nosema* which, if new, would be called *N. Adiei*. A report from Mrs. Adie will appear in the next issue of the Journal.



# SOME NOTES ON INDIAN CALLIPHORINAE.

## PART VI.

### HOW TO RECOGNISE THE INDIAN MYIASIS-PRODUCING FLIES AND THEIR LARVÆ, TOGETHER WITH SOME NOTES ON HOW TO BREED THEM AND STUDY THEIR HABITS.

BY

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ILLUSTRATED

BY

EDITH M. PATTON.

[Received for publication, January 13, 1921.]

DURING the course of my enquiry into the myiasis-producing flies of India, I have had occasion to correspond with medical and veterinary officers, and I find that many of them are anxious to take advantage of the opportunities they have of studying the life histories and habits of these flies. It is very evident, however, that they are not in a position to do so not having had any training in the elements of Entomology. This is much to be regretted, for it is these officers, who see many interesting cases of myiasis in the course of their work, and who could add much to our limited knowledge of this important subject. I have therefore written this paper especially for them, and I trust it will arouse a greater interest in this subject in India, and that many will record their observations on the habits and life histories of the myiasis-producing flies.

#### HOW TO RECOGNISE A FLY.

Before it is possible to study the myiasis-producing species, it is necessary to know how to recognise a fly from other insects, and to

understand its life histories in general so as to be in a position to make accurate observations.

Insects have four wings, two on each side, but all flies have only two, the second pair being represented by curious stalked structures situated below and behind the bases of the wings, and known as the halteres. Like all insects flies have a head, which bears the eyes, the mouth parts and a pair of sensory organs known as the antennæ; a thorax to which are attached six legs, three on each side, and a pair of wings; an abdomen consisting of a number of segments placed one behind the other, the last carrying the external sexual organs.

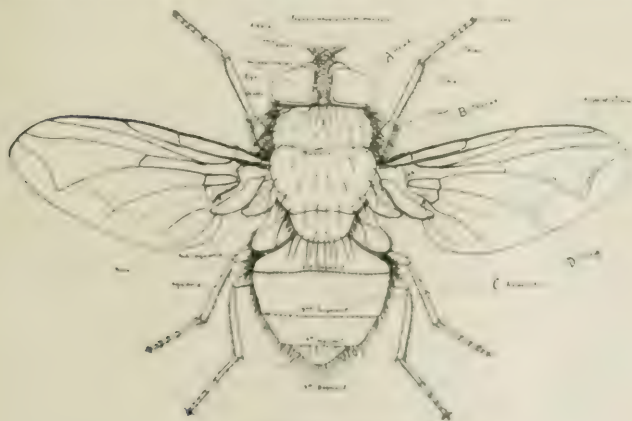
Flies either lay eggs or deposit larvæ, some of which are popularly spoken of as maggots. The eggs are laid either singly, or in a mass of varying numbers, either in, or near the food of the larvæ. On hatching out of the eggs the young larvæ begin to feed, and slowly growing change their skins three or four times, and after each moult certain external structures become better developed, and others appear for the first time. When they reach maturity all growth ceases, and the larvæ become more or less quiescent. In this stage the fly is known as a pupa or puparium, and from which, after a varying time, the perfect insect emerges to live an active life in search of food. The sexes now meet and the female after fertilisation lays its eggs, or deposits its larvæ as the case may be, and the life history begins again. It is important to remember that growth only takes place during the larval stage, and that once the fly hatches out of its puparium it does not grow any more, so that small flies of any species only mean that the larvæ were unable to obtain sufficient food, and on being forced to pupate, could only develop into small specimens.

The order Diptera or Flies is divided into two suborders, the Orthorrhapha and the Cyclorrhapha. The former is again divided into two groups, the Nematocera and the Brachycera. In the Nematocera is included all those simple flies such as mosquitoes, midges and allied forms, most of which are aquatic in their larval stages, the larvæ being specially adapted for living and developing in water, and are provided with various organs for swimming, floating, etc. Well-known examples are the many species of *Culex* and *Anopheles* mosquitoes, and the biting midges, minute dark flies which are such a pest to man and animals in the vicinity of water.

In the Brachycera are included a large number of more robust flies such as the well-known gad flies, which cause such annoyance to animals, and which are believed to carry the parasite of Surt. These flies are often confused with the Bot Flies, but it should be pointed out that they are in no way connected with them. Both the Nematocera and the Brachycera are divided into many families, but as they do not concern us here, they need not be referred to again.

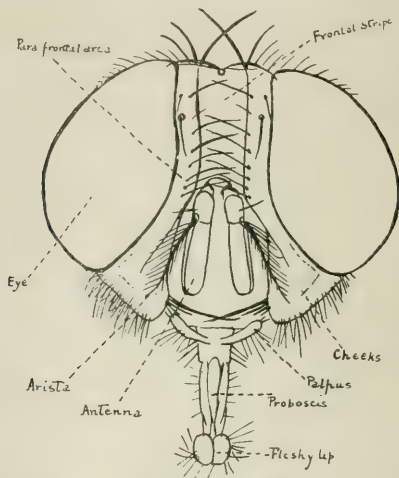
In the Cyclorrhapha are included all the more highly developed flies, and the suborder is divided into three groups, one of which contains the house fly, and allied species. In this group is placed among others two important families, the Muscidae and the Sarcophagidae, the former of which is divided into two subfamilies, the Muscinae and the Calliphorinae. The Muscinae includes all the greyish black flies such as the common house fly, so familiar to everyone; the Calliphorinae contains all the metallic green and blue flies, such as the well known blue and green bottles commonly seen about decaying animal matter. The Sarcophagidae or Flesh Flies contains a large group of greyish striped and chequered flies.

The accompanying line drawing (Text fig. 1) of a female muscid fly will explain the structure of one of these insects. The eyes are



TEXT FIG. 1.—A Typical Female Muscid fly.

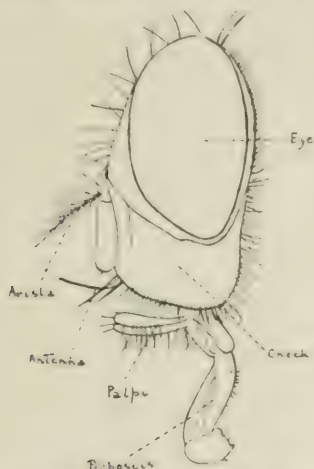
situated at the sides of the head and each consists of hundreds of lenses, and is, therefore, called a compound eye. The whole space between the eyes is the front, and the central dark part bordered with bristles the frontal stripe. (Text fig. 2.) The sexes can always be recognised by noting that the eyes are widely separated in the female, that is, the front is



TEXT FIG. 2.—Front view of head of a Muscid fly.

wide, while in the male the eyes are approximated. The occipital triangle is a small dark area at the top of the head on which is situated the three ocelli or small eyes, amber coloured button-like structures. The area below the eyes on each side and best seen either from the front, or the side, is the cheek. In the middle line there are two approximated elongate, somewhat flat, structures, known as the antennæ, from the basal part of which there projects a long feathered bristle, the arista. The antennæ are the sense organs of the fly and function in much the same way as the human nose. Projecting from the middle line between the cheeks is the mouth of the fly which is an elongated structure known as the proboscis (Text fig. 3); it is easily flexed back and is then almost hidden under the head. At its extremity it is provided with two fleshy lips which have minute channels running towards the opening of the proboscis and through

which the fly sucks up its fluid food. At the base of the proboscis on the upper surface, there are two club-shaped structures known as the palps. The width and colour of the front and frontal stripe, the



TEXT FIG. 3.—Side view of head of a Muscid fly.

colour of the cheeks and the antennae are important points in separating closely related species.

The thorax, the middle segment of the body, is an elongated compact box-like structure, formed of strong plates of chitin, the hard outer skin of the fly, welded together to give better attachment for the powerful muscles which move the wings and legs. The upper surface of the thorax is, in these flies, always provided with strong black bristles arranged in a definite manner, and which are of great use in separating the important species. In this connection, therefore, it is important to note that the thorax is divided into almost two halves by a well marked suture or cleft.

The wings consist of thin membranes stiffened by air tubes which are called veins; it is important to note that the fourth vein (see Text fig. 1) in the Muscidae always bends up towards the third, and this is characteristic of the family. At the base of the wing, there is a large membranous scale, the squama, firmly attached to the thorax,

and which does not move with the wing. Each squama is attached to a folded membrane which in its turn is continuous with the little wing, the alula, which always moves with it. Below the squamæ are the halteres, the representatives of the second pair of wings.

The legs consist of a number of segments jointed together, as shown in text figure 1, the last being provided with a pair of claws.

The abdomen consists of four segments, the first apparent one is, however, in reality the second, the true first segment being more or less suppressed. In the female, the abdomen ends in a long tube, the ovipositor, or egg-laying apparatus, which at rest is telescoped into the last segment. If the observer keeps the text figure before him, he should have no difficulty in recognising a species belonging to the Muscidæ.

It is next necessary to consider the structure of the larva or maggot of this group of flies, a typical member of which is shown in text figure 4.

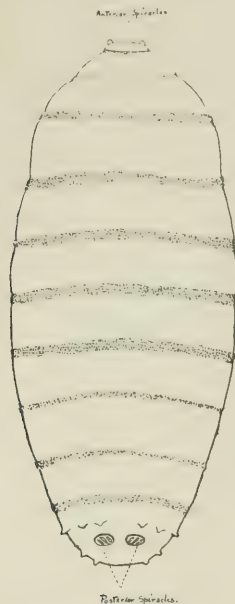


TEXT FIG. 4.—Mature larva of a Muscid fly.

It will be noted that it is elongated consisting of a number of rings or segments thickened at their front ends to form pads which take the place of legs. The front end of the larva is pointed and is provided with a mouth and a pair of long black hooks which are used in progression, and also in fixing it in position. The body segments gradually enlarge from before backwards the hind end being truncated. Every muscid larva possesses two important structures which are of great use in recognising to which species of fly it belongs. At the front end on each side of the apparent third segment there is a small yellowish fan-shaped structure, which, when examined with a high power, is seen to consist of a number of small finger-like protuberances, each with a minute opening at its end. This organ is the anterior spiracle or breathing pore of the larva and internally it is continuous with one of the breathing tubes. The number of processes though somewhat variable in the different species of maggots is sufficiently constant to be of great value in recognising the fly to which it belongs. At the broad end of the maggot, there is another pair of openings which are often mistaken for suckers, known as the posterior stigmatic openings, or breathing pores. Each consists of a round or oval plate of a dark brown colour enclosing three straight or coiled slits; the plates are usually situated close together. Without going into the minute structure of these openings it is sufficient to say that the slits, which are guarded by fine processes, open into the ends of the breathing tubes of the maggot, which are at this point enlarged to hold a relatively large amount of air; the fine processes prevent the entry of any solid matter. The size of the plates, their distance apart, the structure and direction of the slits, and many other important points help to recognise the fly to which the particular maggot belongs. Further the structure of the end of the eighth segment which bears the breathing pores or openings should be noted, for in some larvae the openings are easily seen while in others they are hidden in a hollow formed by the lips of the end of the segment. By noting this point, as I will show further on, it is often possible to locate the species almost at once. In the Muscine, the slits are nearly always coiled, while in the maggots of the Calliphorine they are straight and directed inwards.

When the larva reaches maturity, it always crawls away from the food stuff on which it was feeding and seeks a suitable place in the

earth in which to pupate. A typical pupa, or more correctly a puparium, of a muscid fly is shown in text figure 5.



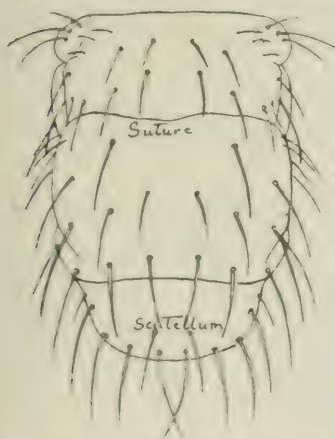
TEXT FIG. 5.—Puparium of a Muscid fly.

It is always barrel-shaped and of a brown colour, and may be mistaken for a seed. It will be noted from its structure that it is merely the hardened larval skin, and the anterior and posterior spiracles can be easily recognised at the ends. If such a puparium is kept in a suitable receptacle, the emergence of the fly can be studied. This remarkable feat is accomplished by the fly pushing off the anterior end of the puparium with the aid of a sac filled with air at the front end of the head. This sac, which is known as the ptilinum, is withdrawn into the head of the fly, when its skin begins to harden. As soon as the fly extricates itself from its puparium, it walks about very actively. Its wings are at first crumpled up but soon begin to unfold and in about 3-4 hours time it is ready to take to flight.

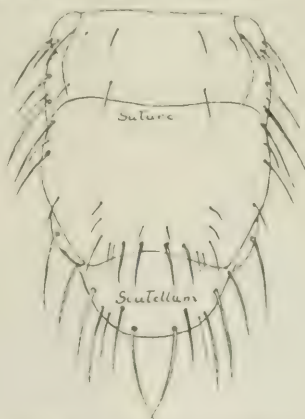
THE IDENTIFICATION OF THE MYIASIS PRODUCING  
FLIES OF INDIA.

The myiasis-producing flies of India belong to two important families, the Muscidae and the Sarcophagidae, the former as pointed out above is again split into two smaller groups, the Muscinae and the Calliphorinae, the latter contains all the important species which concern us here.

The Calliphorinae or Blow Flies are large green or blue insects often with a characteristic brassy sheen. The myiasis-producing species belong to two genera, *Lucilia* and *Chrysomya*, which can be separated by noting that in *Lucilia*, the bristles on the thorax are well developed forming two distinct rows down the middle of the thorax (Text figs. 6 and 7) in front and behind the suture; the thorax, and in most species



TEXT FIG. 6.—Dorsal view of thorax of *Lucilia* to show arrangement of bristles.



TEXT FIG. 7.—Dorsal view of thorax of *Chrysomya* to show arrangement of bristles.

the abdomen is unbanded. In *Chrysomya* on the other hand there are very few bristles on the thorax, and then only one row on each side but instead the surface is covered with downy hairs, the thorax has indistinct longitudinal bands and the abdomen well marked horizontal bands at the bases of the segments. A blue or green myiasis-producing fly with a very bristly thorax is a *Lucilia*, and one with a very

few bristles but a dense covering of fine hairs, and abdominal bands is a *Chrysomyia*. I will first point out how the important species of *Chrysomyia* may be distinguished from each other.

*Chrysomyia bezziana* and *Chrysomyia megacephala* (duRoi) are the two important myiasis-producing Blow Flies of India, the former only breeds in living tissues, while the latter, though occasionally depositing its eggs on sores, wounds, etc., on the bodies of animals, normally breeds in dead bodies, and in decaying organic matter. The females of these two species are very similar in general appearance, and can only be separated by noting the following points:—The eyes of the female *Chrysomyia megacephala* are wider apart than those of *C. bezziana*, that is, the front is wider, more than 1-3rd the width of the whole head, that of *bezziana* is distinctly narrower about 1-3rd or a little less. (Plate LXIII figs. 1 and 2.) The frontal stripe of *megacephala* is slightly wider than that of *bezziana* bulging out a little about its middle. Those portions of the front of *megacephala* nearest the eyes are of a dirty grey colour while in *bezziana* they are distinctly yellower. These differences are well shown in the drawings referred to above. The squamæ of *megacephala* are of a dirty yellow colour while those of *bezziana* are of a waxy white colour. In all other respects the females of the two species are very like each other:

*Chrysomyia nigriceps*, a large blue species which is a common Blow Fly of the Hil. Stations of India, can be recognised by noting that it is very much darker in colour, and that the front of the female is wide and of a dark grey colour. (Plate LXIII, fig. 3.) The frontal stripe is almost black and the cheeks are dark yellow to dark grey with black hairs. The cheeks of the females of *duRoi* and *bezziana* on the other hand are of a bright orange colour with yellow hairs. *Chrysomyia nigriceps* is not known to deposit its eggs in living tissues and is, therefore, not a myiasis-producing species.

*Chrysomyia albiceps* is another important Blow Fly of India, and all Veterinary Officers should be able to recognise it and its hairy maggot. In Australia, this fly is known by the name of *C. rufifacies*, where it is regarded as the most destructive pest to live-stock, as it has within recent years acquired the habit of laying its eggs in soiled wool on the rumps and hind-quarters of sheep, and as a result large quantities of valuable wool, and many sheep are annually destroyed. In India and Mesopotamia, the maggots of this fly are entirely predaceous, feeding on those of other Blow Flies. The female *albiceps* lays its eggs among the

Fig. 1. *Chrysomya*  
*terziaria* ♂ = 11

Fig. 2. *Chrysomya*  
*megacephala* ♀ = 11

Fig. 3. *Chrysomya*  
*microcephala* ♀ = 11

Fig. 4. *Chrysomya*  
*albiceps* ♂ = 11

Fig. 5. *Chrysomya*  
*villanovi* ♂ = 11

Fig. 6. *Chrysomya*  
*marginata* ♀ = 11

Fig. 7. *Lucilia*  
*argyrocephala* ♀ = 11

Fig. 8. *Lucilia*  
*crassa* ♀ = 11

Fig. 9. *Lucilia*  
*pulex* ♀ = 11

Fig. 10. *Lucilia*  
*hirsuta* ♀ = 11

Fig. 11. *Chrysomya*  
*megacephala* ♂ = 11

Fig. 12. *Chrysomya*  
*terziaria* ♂ = 11

Fig. 1. *Ophryomys*  
petraea ♂ × 11

Fig. 2. *Ophryomys*  
mekacephala ♀ 11

Fig. 3. *Ophryomys*  
nitricolor ♀ × 11

Fig. 4. *Ophryomys*  
alpicola ♂ × 11

Fig. 5. *Ophryomys*  
alpinensis ♂ × 11

Fig. 6. *Ophryomys*  
markusovi ♂ × 11

Fig. 7. *Lucilia*  
argyricephala ♂ × 11

Fig. 8. *Lucilia*  
caesia ♀ 11

Fig. 9. *Lucilia*  
bulgarica ♀ 11

Fig. 10. *Lucilia*  
pallardi ♂ × 11

Fig. 11. *Ophryomys*  
mekacephala ♂ × 11

Fig. 12. *Ophryomys*  
petraea ♂ × 11





eggs and larvæ of other species, but chiefly among those of *Chrysomya megacephala*, in decomposing bodies, and the second and third stage larvæ readily attack and suck out the juices of the maggots living with them, and in this way they will clear a dead body of most of the maggots of *C. megacephala*. It is not known whether the maggots of *albiceps* have this habit in Australia, but if they have it, it must be considered a useful fly.

The female can be readily recognised by noting that it is of a brilliant green colour, with a well marked brassy sheen on the thorax and the end of the abdomen. The front is moderately wide (Plate LXIII, fig. 4), the face and cheeks greyish white to silvery white, particularly the latter which are covered with light hairs.

*Chrysomya villeneuvei* is a common Blow Fly in the Nilgiri Hills and at the foot of the Coonoor Ghat. It is seldom seen except on the decomposing bodies of animals. It is of some importance as its second and third stage larvæ are extremely predaceous, even more so than the larvæ of *albiceps*. The female fly has the same breeding habits as those of the female *albiceps*; its third stage larva, which has been fully described in an earlier paper in this Journal, is covered with long processes.

The female fly can be recognised by its brilliant dark green colour, and stout metallic legs. The front is wide (Plate LXIII, fig. 5) the face dirty grey with bluish reflections, and the cheeks are greyish yellow with light hairs; the antennæ are dark. It would be interesting to know the distribution of this fly in India.

*Chrysomya marginalis* is a widely distributed African species, and has been recorded from Quetta; it is probable that it will be found along the North-West Frontier of India. It can be readily recognised by its plum-coloured abdomen, dark patch at the bases and dark band along the front border of the wings. The front in the female is very wide, and of a dark orange colour fading into white at the lower part; the cheeks are light orange with golden hairs (Plate LXIII, fig. 6.)

As pointed out above all the species of *Lucilia* can be recognised by the numerous large bristles on the thorax, the absence of thoracic and in most species, abdominal bands. The most important Indian species is *Lucilia argycephala*, a relatively small bright green fly with bronze reflections. It is a well known myiasis-producing fly in West Africa and Somaliland, its larvæ having been recorded from

the tissues of man and animals; in India, its larvæ occasionally cause myiasis in animals. It normally breeds in the bodies of recently killed animals, and in meat and offal lying about butcheries, and to a certain extent in decaying vegetable matter.

Specimens of this fly can always be obtained from the sweet and meat stalls in all Indian bazars; its characteristic bronze green colour and size make identification easy. The front in the female (Plate LXIII, fig. 7) is wide, the face and cheeks silvery white, and the occipital area greenish; the antennæ are dark.

*Lucilia craggii* is the common Blow Fly of Indian Hill Stations, and is a large species which soon turns purple when dry. It has the habit of entering houses and buzzing round food. It is a most useful species as its larvæ dispose of a large number of unburied bodies. The female front is relatively narrow (Plate LXIII, fig. 8), the frontal stripe almost reaching the eyes, the face and cheeks are dark grey, and the latter are covered with dark hairs.

*Lucilia pulchra* is only of interest from the fact that unlike the common species of Blow Flies which lay eggs, it is viviparous depositing one larva at a time in decomposing animal matter, and in human excrement. It is a handsome fly of a sea green colour with white dusting on the thorax and abdomen. The front is wide in the female (Plate LXIII, fig. 9), the face and cheeks brilliantly white with silky hairs, and the antennæ bright orange. These contrasting colours are so marked that identification is easy.

*Lucilia ballardii* is another South Indian Blow Fly which lays its eggs in decomposing bodies of animals, and is common about fish curing factories. The female front is wide (Plate LXIII, fig. 10), the face and cheeks dirty grey with brown hairs.

Lastly it is important to draw attention to the differences between the male. *Chrysomya megacephala* and *C. bezziana*. In both species, the eyes are closely approximated. (Plate LXIII, figs. 11 and 12.) In the case of *megacephala* there is a well marked area about the middle of each eye, consisting of large lenses, and which is of a bright red colour in life. The lenses of the eyes of the male *bezziana* are all about the same size and are small; the eyes are of a dark red colour. The drawings clearly show these differences.

With the help of these coloured drawings of the front view of the heads of these flies, Veterinary Officers should have no difficulty in identifying the common species, and in particular those which cause

myiasis. Some difficulty may be experienced in distinguishing the female *C. bezziana* from the female *C. mesolephala*, but with a little practice in examining the heads of the two species, and keeping in mind the differences noted above, and shown in the drawings, which are all drawn to the same scale, and from fresh specimens, identification will be made easy.

It is not possible yet to describe in detail the species of *Sarcophaga* whose larvæ cause myiasis in man and animals in India, as these flies have not been bred from their maggots. All the species are extremely like each other, and are large grey flies with dark stripes on the thorax and chequered markings on the abdomen. The species can only be separated by examining microscopic preparations of the external genitalia of the male. I hope that all Medical and Veterinary Officers, who come across the larvæ of these flies in cases of myiasis, both cutaneous and gastro-intestinal, will preserve some of the larvæ in spirit and breed out others, carefully pinning the flies.

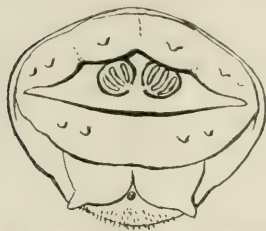
#### THE IDENTIFICATION OF THE LARVÆ OF THE MYIASIS-PRODUCING FLIES OF INDIA.

The short description and illustration of a Muscid larva or maggot given above should enable any observer to make certain he is dealing with the early stages of a fly.

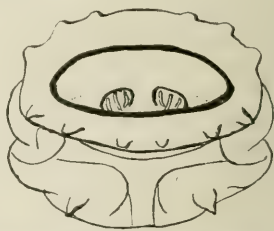
It is first necessary to determine the genus to which the larva belongs. Examine the posterior end of a large specimen with a hand lens, and if the posterior spiracles are comparatively large and enclosed, either in a shallow cleft, or a deep hollow formed by the end of the 8th abdominal segment, the larva is either that of a species of *Chrysomya* or a *Sarcophaga*. Next compress the end from before backwards so as to draw the hipe of the 8th back. In the case of a larva of a *Chrysomya* the posterior spiracles at once become visible, and they can be seen as two large somewhat D-shaped plates, with three brown slits directed downward and inwards. (Text fig. 8.) In *Sarcophaga*, the end of the 8th abdominal segment forms a deep hollow at the bottom of which the spiracular plates are situated (Text fig. 9), and when the end of the larva is compressed, they are only partially visible. Further it will be noted that the outer pair of slits are almost straight, while the inner ones are slightly comma-shaped and directed outwards and downwards. Two other points should be noted. The spines on the segments of the larva of *Sarcophaga* are poorly developed, the larva is much softer, and the anterior spiracles

consist of about ten to fifteen and often more, short finger-like processes. In the larva of a *Chrysomyia* the segmental spines are well developed, and as a rule the anterior spiracles have only a few processes.

If, on the other hand, on examining the end of the larva, it is noted that the posterior spiracles are exposed without compressing the end of the larva, and are not, or only very partially, hidden in a cleft, and in addition are round or slightly pear-shaped, and comparatively small, the larva belongs to a species of *Lucilia*. (Text fig. 10.)



TEXT FIG. 8.—Posterior end of mature larva of *C. bezziana*.



TEXT FIG. 9.—Posterior end of mature larva of *Sarcophaga*.



TEXT FIG. 10.—Posterior end of mature larva of *Lucilia argyricepala*.

Having decided that the larva belongs to a species of *Chrysomyia*, it is next necessary to determine the species, and this can only be done with certainty by examining the posterior spiracles, cleared in caustic potash and mounted on a slide. I have made a number of drawings of the posterior spiracles of the common species of *Chrysomyia* and *Lucilia* from such preparations, and all the figures on Plate LXIV are drawn to scale with a camera lucida and are magnified 80 times. If these drawings are

EXPLANATION OF PLATE LXIV.

Fig. 1. Posterior spiracles of  
*C. albiceps*  $\times 80$ .

Fig. 2. Posterior spiracles of  
*L. craggii*  $\times 80$ .

Fig. 3. Posterior spiracles of  
*C. nigricipes*  $\times 80$ .

Fig. 4. Posterior spiracles of  
*C. bezziana*  $\times 80$ .

Fig. 5. Posterior spiracles  
*C. dux*  $\times 80$ .

Fig. 6. Posterior spiracles of  
*sarcophaga* spl.  $\times 80$ .

Fig. 7. Posterior spiracles of  
*C. villeneuvei*  $\times 80$ .

Fig. 8. Posterior spiracles of  
*L. arg.*

Fig. 1. Posterior spiracles of  
*C. albiceps*  $\times 80$ .

Fig. 2. Posterior spiracles of  
*L. castaneus*  $\times 80$ .

Fig. 3. Posterior spiracles of  
*C. nigriceps*  $\times 80$ .

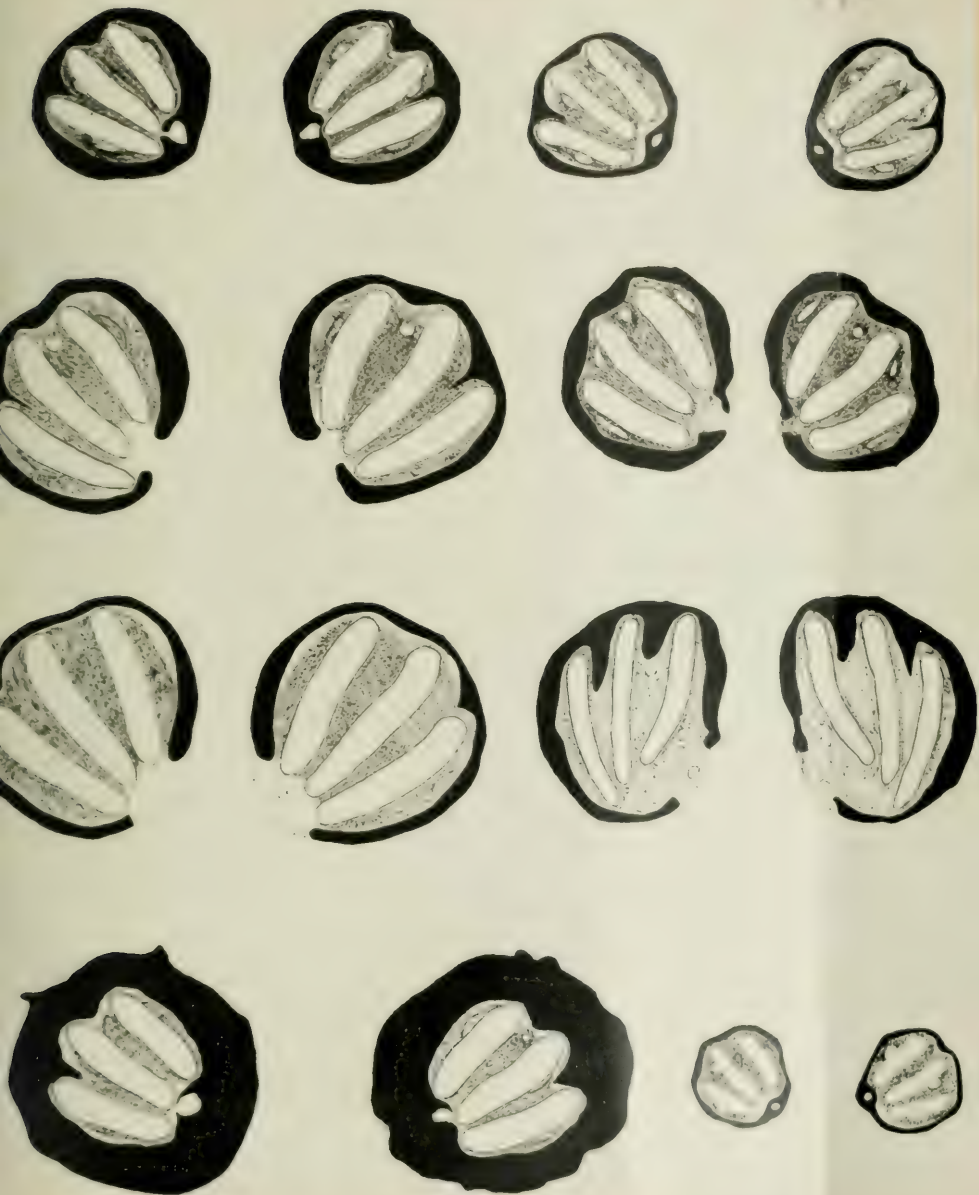
Fig. 4. Posterior spiracles of  
*C. pectoratorius*  $\times 80$ .

Fig. 5. Posterior spiracles  
*C. dux*  $\times 80$ .

Fig. 6. Posterior spiracles of  
*Leptophaea* sp.  $\times 80$ .

Fig. 7. Posterior spiracles of  
*C. vollenweberi*  $\times 80$ .

Fig. 8. Posterior spiracles of  
*L. arcticus*.





carefully examined, and compared with the spiracles, as seen with a hand lens, the observer should have no difficulty in determining the species to which the larva belongs.

The commonest larva which will be found in cases of myiasis in man and animals will be that of *Chrysomya bezziana*, and it is important to be able to distinguish its larva from that of the common bazaar species *Chrysomya megacephala*. The larva of *bezziana* is of a creamy yellow colour, while that of *megacephala* is whiter and softer. The two ends of the larva of *bezziana* are usually much darker, especially the posterior end, than those of the larva of *megacephala*, and the belts of spines are much better developed; the spines of the larva of *megacephala* are not so prominent. The use of these hooks will be made clear if an attempt is made to extract a larva of *bezziana* out of the tissues by pulling on it by its posterior end with a pair of forceps. Considerable force will be required and as often as not the larva will be torn in half. These hooks are, therefore, clearly of use in helping the larva to remain fixed in the tissues in which it is feeding, and especially when in such a situation as the human nose.

Next examine the posterior end of the larva and note that the posterior spiracles are large D-shaped structures, the brown slits directed downwards and inwards, and the strip of chitin surrounding them is wanting at the inner and lower angle. The plates of the larva of *bezziana* are smaller, closer together, and the slits shorter than those of the larva of *megacephala*; the break in the chitin is also smaller. These differences are well shown in figures 4 and 5 on Plate LXIV. The anterior spiracles of the larva of *bezziana* consist of about four or five finger-like processes, while those of *megacephala* always have ten to a dozen. Further it should be noted that there are several breaks in the delicate chitinous membrane between the slits in the case of the larva of *bezziana* while there are none in the case of the larva of *megacephala*.

The larva of *n. grueps* is very like that of *megacephala* and may be mistaken for it, but the posterior spiracles (Plate LXIV, fig. 3) are further apart, and the slits slightly shorter. There is usually one break in the chitin between the inner and middle slit.

There should be no difficulty in recognising the larvae of *Chrysomya albiceps* and *C. villeneuvei*. Both are of a dark grey colour and have long fleshy processes at once distinguishing them from the smooth, somewhat white larvae of the other common Calliphoridae. The processes of the larva of *albiceps* are smooth and have a tuft of dark

spines at their apices, while those of the larva of *villeneuvei* are longer and are covered with spines. These processes have suggested the name 'hairy' often applied to the larvæ of *albiceps*. The posterior spiracles of both species are surrounded by a broad plate of chitin, broader in the larva of *villeneuvei* than in that of *albiceps*; the plates of the former are further apart. (Plate LXIV, figs. 1 and 7.)

The larvæ of species of *Lucilia* are smoother than those of *Chrysomyia*, and, as pointed out above, the posterior spiracles are smaller and are not enclosed in a cleft or slit formed by the end of the 8th segment. They are plainly visible, and it is not necessary to compress the end of the larva to make them more so. The spiracular plates are smaller, more circular or pear-shaped, and the chitin surrounding them of a lighter colour, narrower, and not interrupted.

The posterior spiracles of *Lucilia argyricephala* (Plate LXIV, fig. 8), the most important myiasis-producing species in India, are smaller than those of the larvæ of any of the other species of *Lucilia* I have the opportunity of examining; they are much smaller than those of the larva of *Lucilia craggii* (Plate LXIV, fig. 1), thus affording a very easy means of separating the two.

It should be remembered that the second stage larvæ of species of *Chrysomyia*, *Lucilia* and *Sarcophaga* can always be recognised by noting that there are only two slits in the posterior spiracles instead of three.

The posterior spiracles of the mature third stage larva of the common myiasis-producing Indian species of *Sarcophaga* are shown in figure 6 of the Plate, and it will be seen that the slits are longer, narrower, and run in a different direction to those of the larvæ of *Chrysomyia* or *Lucilia*.

The larva of *Aphiochaeta* can be recognised by their small size, broad somewhat flattened segments, and by their peculiar method of progression which suggests that of some butterfly larvæ.

#### HOW TO BREED THE INDIAN BLOW FLIES.

I have already pointed out that the larvæ of *Chrysomyia bezziana* cannot be reared to maturity by placing them in meat, or the dead body of an animal. They will only live in living tissues and therefore it is useless attempting to breed them in any inert organic matter. They can, however, be easily raised if they are placed in the wound on the body of an animal. The larvæ of *Chrysomyia megacephala*, *Lucilia argyricephala*,

*Sarcophaga* and *Aphrocharta* can, on the other hand, be easily reared to maturity by placing them in decaying meat, etc. In carrying out such experiments it should be remembered that when the meat in which they are placed begins to decompose, other Blow Flies, and particularly species of *Sarcophaga*, will be attracted to it, and if precautions are not taken to exclude them from laying their eggs or depositing their larvae in the meat or dead body in which the myiasis-producing larvae were placed, it will soon be found that the latter have disappeared, or have become so mixed up with the hundreds of other larvae that it will then be impossible to find them.

I have placed meat and the dead bodies of animals, containing special larvae in various receptacles, such as jars, bottles with screw tops, etc., but it was soon found that it was impossible to exclude the larvae of other Blow Flies and in particular those of *Sarcophaga*. When the meat is placed in a screw-top bottle on some sand, and the lid perforated with small holes, I found that the female *Sarcophaga* was able to drop her larvae either through the holes, or to deposit them near the hole, and later they were able to find their way in; and once the larvae of *Sarcophaga* get into the meat, they will soon destroy the myiasis-producing larvae which one is trying to rear. Glass jars were covered with glass lids, the edges carefully vaselined, yet in spite of this precaution, and many others, the larvae of *Sarcophaga* eventually reached the meat. After trying all these methods I soon gave them up and then tried another, long ago recommended by Fabr<sup>e</sup> of enclosing the meat in numerous rolls of newspapers or in a paper bag. It will be remembered that Fabr<sup>e</sup> suggested that the game exposed for sale in meat shops, and particularly pheasants and partridges may be protected from the attention of *Calliphora erythrocephala* and *C. canadensis* by enclosing each bird in a paper bag. Such bags are an effective barrier and no Blow Fly larvae can possibly get into the contents. In this way I have been able to breed out all the common Indian Blow Flies and to study their early stages. When one is dealing with decomposing meat or a dead body great care should be taken to add fresh papers when the outer layer becomes soaked with the fluid from decaying meat or body. If this precaution is not taken, other flies will lay their eggs under the paper and the first stage larvae will soon work their way through the wet paper which becomes in time more or less dissolved.

If this method of breeding out any of the larvae of the Blow Flies is adopted, no difficulty will be experienced in obtaining all the stages from

the egg to the adults. I have laid out the dead bodies of rabbits and watched the females lay their eggs, these were then transferred to another fresh body, which was then carefully wrapped up in numerous rolls of paper. It is then quite easy to collect the first, second and third stage larvæ, the puparia and then to hatch out the flies. As soon as the larvæ reach maturity, they endeavour to crawl out of the rolls of paper, as they always prefer to pupate in earth. When it is noted that they are nearly mature, extra precautions should be taken to prevent them from getting out and crawling away. Fresh paper should be added, and the whole carefully tied on. It will then be found that they will pupate in the rolls and the puparia can later be removed and placed in tubes, not more than two or three in each. In a few days the flies will hatch out and they should be left for 24 to 36 hours in order to allow their wings to stretch, and their bodies to harden. They should now be transferred to a killing bottle and later pinned.

Specimens of mature larvæ when recovered from cases of myiasis should be preserved by dropping them in boiling water; they stretch out, and later they can be placed in a tube with 80 per cent alcohol in which they harden. Some of the puparia should also be placed in the spirit with the larvæ. When the flies hatch out, they should be pinned along with their puparia.

It is of the utmost importance to try and rear all specimens of *Sarcophaga* larvæ, first preserving a few in alcohol. The majority should be placed on a piece of meat which should be wrapped up in rolls of paper, as described above, and the utmost precautions should be taken to prevent any other larvæ from getting into the meat or body. If this were done in every case of myiasis caused by the larvæ of *Sarcophaga*, we would soon be in a position to recognise their larvæ and to determine the species accurately. It should be remembered that the species of *Sarcophaga* can only be determined by examining the males and it is therefore important to pin as many specimens of the adults as possible.

The larvæ of *Aphiochaeta* can be easily bred in decaying meat, or in the body of a dead insect. Here again, larvæ, puparia, and adult flies should be preserved in 80 per cent alcohol; it is always better to place the flies in spirit than to attempt to pin them owing to their small size.

The larvæ of the *Oestridæ*, and there are not many in India, can always be recognised by their large size, squat appearance, soft folded skins, and by the well developed black spines on their bodies. They are

found either under the skin (*Hypoderma*) of animals, such as goats, and cattle, in the nasal cavities (*Oestrus ovis* in sheep and goats and *Cephalopais tallator* in the camel), or in the stomachs of horses (*Gasterophilus intestinalis*), and elephants (*Cobboldia elephantis*). It should always be remembered that these larvæ when squeezed out of the skins of their hosts, or when removed post-mortem from the nasal cavities and stomach will never pupate, but invariably die when placed in earth no matter what care is taken; this is simply due to the fact that they are not mature, and are therefore not ready to leave the animal host and pupate. All larvæ removed in this way should be at once preserved in alcohol, it is mere waste of time trying to keep them alive.

These Bot and Warble fly larvæ, when mature, leave their hosts either by crawling out of the skin, nasal cavities or are passed out in the excreta of the animal. Such larvæ if placed in a cigarette tin in some damp earth, will pupate; they should, however, be handled as little as possible, and it is best to fill the tin with earth and place them on it, and allow them to burrow in. Small air holes should be made in the lid of the tin and it should be placed in a dark place where no ants, etc., can reach the larvæ. As soon as they have contracted up and hardened their outer skins becoming dark brown, now place them in a tin with a little earth and watch them for the flies to hatch out. These should be transferred to a tube and allowed to harden and then can later be pinned with their puparia.

Veterinary Officers in India have many opportunities of studying the myiasis-producing flies and their larvæ. I trust that those who have to deal with elephants will endeavour to work out the life history of *Cobboldia elephantis*, and collect the larvæ from the excreta of the elephant and hatch out the flies. I will be glad to identify any material bearing on this subject, and will give any officer who is interested in the subject any further information. The material should be sent to me at the Zoological Department, Edinburgh University.

## SOME NOTES ON INDIAN CALLIPHORINAE.

### PART VII.

#### ADDITIONAL CASES OF MYIASIS CAUSED BY THE LARVÆ OF CHRYSOMYIA BEZZIANA VILL., TOGETHER WITH SOME NOTES ON THE DIPTERA WHICH CAUSE MYIASIS IN MAN AND ANIMALS.

BY

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[Received for publication, January 13, 1921.]

IN Part I of this series of papers I described and illustrated in some detail *Chrysomya bezziana* Vill., in all its stages, and gave a few notes on 40 cases of myiasis in man and animals caused by its larvæ. It will be remembered that I pointed out that this Calliphorine only breeds in living tissues, and is therefore one of the specific myiasis-producing flies of India. It is widely distributed in India, Burma, Assam and Ceylon and is found at altitudes of 6,000 feet.

Previous to these observations, *C. bezziana* was only known from the Ethiopian Region having been recorded from the Upper Ivory Coast by Bouet and Roubaud, from the Belgian Congo by Rovere, and from French Upper Guinea by Joyeux. In Africa, according to these observers, the larvæ of this fly cause myiasis in the larger animals, chiefly cattle, though Joyeux records them from a case of myiasis in a horse.

Rovere has described the life history of *bezziana*. He states that the female fly lays from 70 to 95 eggs on the skin of cattle where there is some mucus. The larvae hatch in from 18 to 24 hours, penetrate the unbroken skin down to the subcutaneous tissues and there undergo their development; when mature they crawl out and pupate in the ground.

Rovere's observations on the method of oviposition, and the number of eggs laid by the female *bezziana*, do not, however, accord with mine in India. I have already recorded the finding of a mass of 486 eggs of this fly on a piece of lint on a foul ulcer (Part I, Case 10). Since then I have had sent me a mass of 522 eggs which were laid on an open sore on the left elbow of a patient (Case 41 below), and all the first stage larvae which had hatched out from a mass of eggs laid on a sore on the hip of a bull calf (Case 59). The mass of eggs was removed from the sore and placed in a tube and when the larvae hatched the tube was filled with 80 per cent alcohol.

These observations and specimens clearly demonstrate the fact that the female *bezziana* in India lays many hundreds of eggs all in a mass directly on the diseased tissues. I think, therefore, that there can be very little doubt that the small number of eggs recorded by Rovere can only mean that the fly was disturbed in the act of oviposition, and did not lay the full number of eggs. Further it should be noted that Rovere states he found the eggs on the unbroken skin, a most unusual site. If the female *bezziana* lays its eggs on the unbroken skin of animals, there should be no limit to its numbers in Africa, and it should be a much more serious pest than it is known to be at present.

In India *bezziana* only lays its eggs on, or near, diseased tissues, being attracted by the odour of a foul discharge, on which the fly most probably feeds. I have little doubt that the observations recorded by Rovere represent some unusual method of oviposition of *bezziana* in Africa and that there, as in India, it usually lays its eggs on some pre-existing sore. The observations of Roubaud and van Soestghem support this view for they found that at Zimbe in the Belgian Congo, the larvae of *bezziana* were always found in a pre-existing sore. It therefore seems highly desirable that further observations on the life history of *bezziana* should be made in Africa.

Although *bezziana* has up to the present not been recorded from Central, South, or East Africa, I believe the larvae recorded by Jack

from myiasis in cattle in Rhodesia belong to this fly. Aders records its larvæ from sores in cattle in Zanzibar. It is of some interest to note that the larvæ of *bezziana* have only been recorded from animals in Africa, and as far as I am aware they have never been found in man. In India, on the other hand, *bezziana* appears to be the only Calliphorine which causes myiasis in man, and it is extremely common.

Turning now to India and adjacent parts, I find that there are many records of the larvæ of *bezziana* from human cases of myiasis. Most of them have been recorded in the *Indian Medical Gazette*, and unfortunately I am unable at present to consult a complete set of this journal, and can only refer to a few of the cases, some of which are, however, worth noting. It should be mentioned that *bezziana* in India has been confused with *Cochliomyia macellaria*, *Chrysomyia megacephala* and *C. flaviceps*. There can be very little doubt from a perusal of the descriptions of the larvæ and the illustrations accompanying these cases, that in each case the observer was dealing with *Chrysomyia bezziana*.

Powell records five cases of myiasis of the nose and accessory sinuses caused by the larvæ of *bezziana*; three of the cases proved fatal. Chetti records a case of nasal myiasis in an adult male from Bassein, Burma. The crude drawings of the larva leave no doubt that it is that of *bezziana*. Patterson records a remarkable case from Tezpur, Assam, in a debilitated cooly woman which proved fatal. The whole of the nose sloughed away and the eyes were destroyed before death. Large numbers of larvæ were collected from the nose and accessory sinuses. The description and figures of the larva exactly correspond to those of *bezziana*.

Cameron records two cases of nasal myiasis from the Punjab and his description of the larva shews that it was that of *bezziana*. Lastly Riley records two cases of myiasis from Behar, the larva and the fly are described by the late Mr. Howlett, and his descriptions leave no doubt that the fly was *Chrysomyia bezziana*. Riley is wrongly under the impression that the fly is viviparous. Further he states that, 'when grown experimentally on decomposing meat, placed on dry sand, the young larvæ were noticed to feed ravenously on the meat soon after birth and to grow with amazing rapidity until they were fully developed.' In this connection it should be noted that the author does not mention how he excluded other Blow Flies from ovipositing on the meat, and in particular in preventing *Sarcophaga*

from depositing its larvæ. In my own experiments with the first stage larvæ of *bezziana*, I failed to rear a single one to maturity, and they all died, even though some were placed in the body of a recently dead rabbit. Nor have I succeeded in rearing the second stage larvæ by placing them in the bodies of freshly killed rabbits, in fresh meat, or in the decomposing bodies of rabbits. Further, in these experiments it was extremely difficult to exclude other Blow Flies from depositing their eggs near and on the meat, etc., and almost impossible to prevent the larvæ of *Sarcophaga* from reaching it, no matter in what sort of receptacle the meat or rabbit's body was placed. I have already pointed out that the only way to breed any particular larva is by placing the meat or body in numerous rolls of paper. I am, therefore, forced to doubt Riley's observations on the rearing of first stage larvæ of *bezziana* in decomposing meat. I have never been able to breed *bezziana* in nature by exposing freshly killed animals, fresh meat, or decomposing bodies, nor have I ever seen the fly in nature. It is true I have reared a few small third stage larvæ by placing them in meat and the bodies of rabbits, but here it was noted that the larvæ made no attempt to feed, but soon crawled into the sand under the body or meat and pupated. The puparia were always small, and from the majority no flies hatched. In my opinion *Chrysomya bezziana* never breeds in any decomposing animal matter in nature, in India, but only in living tissues.

These represent some of the more important records of cases of nasal myiasis in India caused by the larvæ of *bezziana*. I hope on another occasion to collect all these cases together and to publish a complete list.

I will now give some short notes of a further series of cases of myiasis in man and animals from which I have had the larvæ or puparia of *bezziana* sent me. The numbers are continued from Part I of this series of notes.

#### HUMAN MYIASIS.

*Case 25.*—A number of dead larvæ were sent by Sub-Assistant Surgeon Khumar Prasad, Rihel, Sanger District, C. P., from a sloughing ulcer on the external orbit of a female patient.

*Case 26.*—A number of young third stage larvæ in alcohol were sent by Sub-Assistant Surgeon Ram Chand, Jajpur Dispensary, Rhotak, Punjab, collected from the internal ear of a youth aged 18. The patient

said an ant had crawled into his ear, and he had it syringed out by a native doctor, when a quantity of blood-stained fluid came from the ear. On examination 15 days later there were many larvæ in the internal ear. The patient complained of a creeping sensation in the ear.

*Case 27.*—A number of living third stage larvæ were sent by Sub-Assistant Surgeon Ram Chand, Jajjar Dispensary, taken from the nose of a female patient aged 30. The patient stated that 11 days previously she had a discharge from the nose, and that flies sat on and around the nose. Her whole face was swollen, and there was a purulent discharge from the nostrils. Many maggots were removed from the nose and several crawled out of their own accord.

*Case 28.*—A number of living larvæ, and several preserved in spirit, were sent by Dr. Asana, Civil Surgeon, Kaira, Bombay, taken from an abscess on the scalp of a girl aged 10 years.

*Case 29.*—Several living larvæ, and others preserved in spirit, were sent by Major H. P. Cook, Civil Surgeon, Moradabad, U. P., collected from a septic wound on the face of a patient.

*Case 30.*—A number of puparia of *bezziana* were sent by Dr. S. Subba Rao, District Medical Officer, Shimoga, Mysore, the larvæ having been collected from a sloughing ulcer on the scrotum of a patient.

*Case 31.*—Some living larvæ and others preserved in spirit were sent by Sub-Assistant Surgeon Sanjiva Rao, Local Fund Dispensary, Valavanur, from a chronic ulcer on the right temple of a female child aged 1 year. The ulcer was of two months' duration, and had a large pocket on its lower and front aspect in which the larvæ were found.

*Case 32.*—A number of hatched flies and puparia, as well as larvæ in spirit, were sent by Sub-Assistant Surgeon Jivan Lal, Jampur Dispensary, Dehra Ghazi Khan District, Punjab, the larvæ having been collected from a sloughing ulcer on the penis of a boy aged 6 years. The boy had been circumcised by a native quack 15 days before. The wound became infected and a sloughing ulcer formed at the end of the penis; flies were said to have swarmed around it, and a female *bezziana* must have laid its eggs on the ulcer. When admitted to the hospital, the patient had a foul smelling ulcer involving the whole of the penis, and extending 1" into the surrounding tissues. The scrotum and pubis were œdematous, and the soft parts around the end of the penis destroyed; the urethra, however, was intact. The ulcer was full of larvæ of *bezziana*, and several hundreds were removed.

*Case 33.*—A number of puparia of *bezziana* were received from the Superintendent of the King George's Hospital, Lucknow. The larvæ were removed from the nose of a male patient aged 45.

*Case 34.*—A number of dead larvæ, and two flies which had hatched in the parcel, were sent by Assistant-Surgeon H. P. Datta, M.B., Mut. Hospital, Narsinghpur, C.P., collected from the vagina of a female patient who complained of difficulty in micturition.

*Case 35.*—A number of puparia of *bezziana* were received from Sub-Assistant Surgeon Peltam, Hissar Dispensary, Hissar District, Punjab, the larvæ having been collected from the nose of a patient.

*Case 36.*—A number of larvæ preserved in spirit were sent by Rao Bahadur W. V. Kane, L.M. & S., Civil Surgeon, Nimar, Khandwa, C.P., collected from the nose of a patient suffering from ozena.

*Case 37.*—A number of living larvæ of *bezziana* were sent by Rao Bahadur W. V. Kane, L.M. & S., collected from a sloughing wound in the perineum, probably following an abscess. It is interesting to note that the larvæ of *Aphiochaeta xanthina* were also sent with the larvæ of *bezziana*.

*Case 38.*—A number of dead larvæ were received from Dr. S. Subba Rao, District Medical Officer, Shimoga, Mysore, collected from a sloughing ulcer on the dorsum of the right foot, near the great toe, of a patient aged 30.

*Case 39.*—Fifteen puparia of *bezziana* were sent by Dr. Asani, Civil Surgeon, Kurr. Bombay, the larvæ being removed from the nose of a female patient.

*Case 40.*—A number of puparia, some flies, and specimens of larvæ preserved in alcohol, were sent by Dr. Syed Mohammed Shaffigur, Medical Officer in charge of Arwal Dispensary, Gaya District, Bihar and Orissa, taken from a gangrenous sore on the knee of an inmate patient aged 25; the sore had been left without a dressing for some time.

*Case 41.*—A mass of 522 eggs of *bezziana* preserved in alcohol were sent by Major S. Singh, I.M.S., Civil Surgeon, Jullundur, removed from an open sore at the feet of a patient.

*Case 42.*—A few dead larvæ of *bezziana* were sent by Major D. Sherston-Baker, I.M.S., Medical Officer in charge of King Edward VII Memorial Hospital, Secunderabad, Deccan, collected from a case of cancer of the cheek.

*Case 43.*—Some living larvæ, others preserved in alcohol, and several puparia, were sent by Sub-Assistant Surgeon G. Ramalingam, Secunderabad, Deccan, collected from a case of cancer of the cheek.

Bellary, the larvæ having been collected from an ulcer on the middle of the left frontal bone of a female child aged 8 years.

*Case 44.*—A number of puparia were received from Major F. W. Summers, I.M.S., Civil Surgeon, Cawnpore, U.P. The larvæ were collected from an ulcer on the body of a patient.

*Case 45.*—A number of dead larvæ and puparia were received from the Civil Surgeon, Aligarh, U.P., the larvæ having been collected from the mouth of a patient who had gingivitis, and who had to keep his mouth open. A female *bezziana* had evidently oviposited on the gums.

*Case 46.*—A number of young third stage larvæ were received from Major C. A. Gourlay, Superintendent, Campbell Medical School and Hospital, Calcutta, collected from a septic ulcer on the foot of a patient.

*Case 47.*—A number of dead third stage larvæ were received from Dr. A. Reid, Mokameh Dispensary, Patna, Bihar, collected from an unhealthy sore on the great toe of a man aged 40 years.

*Case 48.*—A number of dead third stage larvæ were received from the Medical Officer in charge, King Edward VII Hospital, Benares, collected from the nose of a male patient aged 60, who had a purulent discharge from his nose for a long time. It was a typical case of nasal myiasis with swelling of the nose and accessory sinuses.

*Case 49.*—A few dead larvæ were received from Sub-Assistant Surgeon Ali Mohamed, Phalia Dispensary, Gujarat District, Punjab, collected from the external ear of a girl aged 6 years, who had recently had them pierced for ear-rings.

*Case 50.*—A few dead larvæ were received from Sub-Assistant Surgeon M. A. Khan, Gopalganj, Saran District, Bihar and Orissa, collected from a sore on the thumb of a patient.

*Case 51.*—A male *C. bezziana* was sent by Major Froilano de Mello, Professor of Bacteriology, Medical School, Novo Goa, Portuguese India, bred out from a larva obtained from a large syphilitic ulcer on the right malleolus of a patient in the Military Hospital. The ulcer spread across the foot to the other malleolus. The tissues were extensively destroyed, almost to the bone.

*Case 52.*—A number of living larvæ were sent by Sub-Assistant Surgeon Syed Ahmed Ali, Hatta Dispensary, Damoh District, C.P., collected from an ulcer on the scalp of a boy.

*Case 53.*—A number of living larvæ of *bezziana* were sent by the same observer from a large ulcer on the hip of a pauper who died 5 days after admission.

*Case 54.*—A number of puparia were received from Lieut.-Col. Murison, I.M.S., Civil Surgeon, Belgaum, Bombay, the larvæ having been collected from a septic wound around the eye of a patient.

*Case 55.* A number of larvæ of *bezziana*, preserved in alcohol, were sent by Sub-Assistant Surgeon P. Audinarayanamurthy, Local Fund Hospital, Koraput, collected from an extensive burn on the chest of a woman aged 30.

*Case 56.* A second stage larva of *bezziana*, mounted on a slide, was given me by Mr. Senior White, F.E.S., Matale, Ceylon. The larva had been removed from the internal ear of a child in Matale Hospital, Ceylon.

*Case 57.*—A number of small living third stage larvæ of *bezziana* and some mature larvæ of *Aphiochaeta xanthina* were sent by Sub-Assistant Surgeon Boishab Charan Salen, Phulbani, Orissa, collected from an ulcer on the scalp of a small boy. A pimple which had burst was left without treatment, with the result that the females of *bezziana* and *xanthina* deposited their eggs on the ulcer.

*Case 58.* Larvæ of *bezziana*, preserved in spirit and formalin, and others alive, were sent by Major R. E. Wright, I.M.S., Superintendent, Government Ophthalmic Hospital, Madras, collected from an ulcer in the region of the frontal sinuses and orbit of a female patient aged about 30. This case has been fully recorded in the *Indian Medical Gazette*.

*Case 59.*—A large number of mature larvæ of *bezziana* preserved in spirit, and a number which had pupated on the journey, were sent by Captain J. A. F. Harvey, I.M.D., Civil Surgeon, Damoh. The larvæ were collected by the Assistant-Surgeon, Main Hospital, Damoh, Central Provinces, from septic wounds on the head, face, and ear of a woman who had been attacked by a wild bear on the 17th December, 1920. The bear had evidently torn off the nose and most of the cheeks exposing the bones, the wound communicating directly with the frontal sinuses. A female *bezziana* had been attracted by the septic nature of the wounds, and the larvæ made their appearance on 26th December, 1920.

#### ANIMAL MYIASIS.

*Case 17.* A number of puparia were received from Veterinary Assistant L. Audinarayanasaami, Russelkunda, Coimbatore District.

Madras, the larvæ having been collected from a sore on the back of a bull buffalo.

*Case 18.*—Puparia were received from the same observer, the larvæ of which were collected from a cancerous ulcer on the vulva of a cow.

*Case 19.*—One dead larva and several puparia were received from Mr. B. B. Joshi, Veterinary Officer in charge, Public Veterinary Hospital, Bhamburda, Poona. The larvæ were collected from sores on a cow.

*Case 20.*—A number of living larvæ, and others preserved in alcohol, were received from Veterinary Assistant A. C. Rantna, Cuttack, Bihar and Orissa, collected from the base of a broken horn of a cow.

*Case 21.*—A number of puparia were received from Veterinary Assistant M. A. Merchant, Surat, Bombay. The larvæ were collected from an ulcer on the neck of a bullock.

*Case 22.*—A number of living larvæ and puparia were received from N. Muniappa, Veterinary Officer, Veterinary Hospital, Saidapet, Chingleput District, Madras. The larvæ were collected from a wound following the removal of a fibrous tumour on the neck of a bullock.

*Case 23.*—A number of living larvæ were received from the Veterinary Assistant, Veterinary Hospital, Negapatam, Tanjore District, Madras, collected from a wound on the body of a bullock.

*Case 24.*—Two living larvæ, others dead, and several puparia, were received from Mr. B. B. Joshi, Veterinary Officer in charge, Public Veterinary Hospital, Bhamburda, Poona, Bombay. The larvæ were collected from wounds on cattle brought for treatment to the hospital.

*Case 25.*—Living and preserved larvæ, as well as puparia, were received from Veterinary Assistant M. G. Kulkarni, Belgaum. The larvæ were collected from a wound on the body of a dog.

*Case 26.*—A large number of dead larvæ and living puparia were received from the Veterinary Assistant, Cuddalore, South Arcot District, Madras. The larvæ were collected from the vagina of a cow; they had burrowed deeply into the vaginal wall.

*Case 27.*—A number of dead larvæ were received from Veterinary Assistant R. Jayaram, Bezwada, Kistna District, Madras, collected from a wound on the neck of a bull following the removal of a tumour.

*Case 28.*—A number of third stage living larvæ and many second stage in spirit were received from T. R. Khildar, Veterinary Assistant, Pandharpur, Sholapur District, Bombay, collected from the eyeball of a cow. The animal had a papilloma of the eye which evidently ulcerated

and attracted a female *bezziana*. The larvæ burrowed into the eyeball, completely destroying it.

*Case 29.*—A number of second stage larvæ of *bezziana* in spirit were sent by Mr. B. B. Joshi, Poona, collected from the broken horn of a bullock.

*Case 30.*—A number of living third stage larvæ were received from Veterinary Assistant C. V. Dixon, Veterinary Hospital, Ahmedabad, collected from a wound near the anus of a dog.

*Case 31.*—A number of larvæ, preserved in spirit, were sent by Veterinary Assistant B. B. Patel, Bulsar, Surat, Bombay, collected from wounds on a cow and from the penis sheath of a horse.

*Case 32.*—A number of dead larvæ received from Veterinary Assistant Viswanath Sastri, Berhampore, Ganjam, Madras, collected from a girth wound on an aged donkey.

*Case 33.*—Two dead larvæ of *bezziana* were received from Veterinary Assistant M. R. Tagore, Savada, East Khandesh, Bombay, taken from a wound on the neck of a bullock. It is interesting to note that a number of larvæ of *Aphiochaeta xanthina* were sent from the same case.

*Case 34.*—A number of dead larvæ, others preserved in spirit, and a number of dead specimens of adults of *bezziana*, were received from the Veterinary Assistant, Godhra, Panch-Mahals District, Bombay, collected from a wound on the chest of a heifer.

*Case 35.*—A number of dead larvæ and puparia of *bezziana* were sent by Veterinary Assistant J. Stewart, Purulia, Bihar and Orissa. The larvæ were collected from an open wound on the body of a bullock.

*Case 36.*—A number of mature living larvæ were received from Y. K. Shetty, Veterinary Assistant, Baramati, Poona, collected from a wound on the external surface of the right thigh of a dog.

*Case 37.*—A number of larvæ were sent by Veterinary Assistant, Dharwar, Bombay, collected from a wound on the body of a buffalo.

*Case 38.*—A number of dead larvæ were received from the same observer, from a wound on the body of a cow.

*Case 39.*—Dead larvæ were sent by the same observer from a wound on the body of a cow.

*Case 40.*—A number of living larvæ were sent by the same observer from a yoke gall on a bullock.

*Case 41.*—A number of puparia were received from the same observer, the larvæ having been collected from a wound on the leg of a cow buffalo.

*Case 42.*—A number of dead larvæ were received from the Veterinary Assistant, Pandharpur, Sholapur District, Bombay, collected from the vulva of a cow. It was noticed that the animal had a wound on one of the lips of the vulva, this later ulcerated and contained many larvæ of *bezziana*.

*Case 43.*—A number of larvæ, preserved in spirit, were received from Veterinary Assistant V. R. Chatuphale, Dahannu Thana, Bombay, collected from the near hind heel of a bullock.

*Case 44.*—A number of dead larvæ were sent by Mr. B. B. Joshi, Poona, collected from a wound on the body of a bullock.

*Case 45.*—A number of puparia and dead larvæ were sent by the Veterinary Assistant, Hubli, Dharwar, Bombay, the larvæ were collected from a wound on the withers of a pony.

*Case 46.*—A number of larvæ, preserved in alcohol, were received from Veterinary Assistant G. V. Dadhe, Jalgaon, West Kandesh, Bombay, collected from a wound on the inner side of the thigh of a bull-dog.

*Case 47.*—A number of dead larvæ were received from Veterinary Assistant D. B. Mamtara, Bandra, Thana, Bombay, collected from a broken horn of a buffalo.

*Case 48.*—A number of puparia and dead larvæ were received from the Veterinary Assistant, Pandharpur, Sholapur, Bombay, collected from the base of a broken horn of a bullock. The larvæ were found in the wound 15 days after the horn was broken.

*Case 49.*—A number of puparia were received from Veterinary Assistant M. T. Krishnaswami, Virakeralampudur, Tinnevely District, Madras. The larvæ were collected from an unhealthy ulcer in the nostril of a bullock near the nose string hole.

*Case 50.*—Two puparia and larvæ in spirit were sent by Sub-Assistant Surgeon H. Govinda Gudiyar, L.M.P., Local Fund Dispensary, Talavadi, Coimbatore, Madras. The larvæ were collected from an ulcer on the inner aspect of the thigh of a bull near the scrotum.

*Case 51.*—A number of larvæ, preserved in spirit, were sent by Veterinary Assistant J. E. D. Sigamony, Madanapalli, Chittoor, Madras, collected from the vagina of a cow.

*Case 52.*—A number of hatched flies were received from Mr. B. B. Joshi, Poona. The larvæ were collected from a wound on a cow.

*Case 53.*—A number of dead larvæ were sent by the Touring Veterinary Assistant, Tanjore District, Madras, from the ear of a bullock which had recovered from an attack of rinderpest.

*Case 54.*—A number of dead larvæ were sent by the same observer from the ear of another bullock.

*Case 55.*—A number of dead and some living ones were sent by the same observer from a wound on the body of a cow.

*Case 56.*—Some dead larvæ, and two second stage larvæ in spirit, were received from P. S. Sundaram, G.B.V.C., Officer in charge of the Veterinary Hospital, Pachmari, C.P., collected from a deep wound on the scrotum of a dog.

*Case 57.*—A number of larvæ, preserved in alcohol, were sent by Veterinary Assistant A. C. Patel, Surat, Bombay, collected from a sore which followed a blister applied for sesamoiditis.

*Case 58.*—A number of dead larvæ were received from P. S. Sundaram, Officer in charge of the Veterinary Hospital, Pachmari, collected from a sore on the glans penis of a horse.

*Case 59.*—All the larvæ which hatched out of a mass of eggs of *C. bezziana* laid on a sore on the hip of a bull calf, together with the fly which laid the eggs, were sent by Mr. B. B. Joshi, Poona, who caught the fly himself. This is the first occasion in which *bezziana* has been caught in India laying its eggs.

*Case 60.*—A number of larvæ of *bezziana* were sent by N. Subramani, Veterinary Officer in charge of the Veterinary Hospital, Sivagiri, Tinnevely District, Madras, collected from a neglected wound in the nostril of a bullock caused by a nose string.

*Case 61.*—A number of larvæ, preserved in alcohol, were received from the Veterinary Assistant, Nadiad, Kaira, Chapra, Bombay, collected from a sore on the umbilicus of a kid.

*Case 62.*—A number of puparia and larvæ, preserved in alcohol, were sent by Veterinary Assistant Y. K. Shetty, Batamati, Poona District, Bombay, collected from a wound on the vulva of a buffalo.

*Case 63.*—A large number of second stage larvæ, and the egg shells of the same, were sent by Veterinary Assistant V. K. Chatuphate, Dahann, Thana, Bombay, collected from a wound on a cow buffalo.

*Case 64.*—A number of hatched *bezziana* were sent by Veterinary Assistant D. M. Gadkari, Satara Veterinary Hospital, Bombay, the larvæ were collected from a wound at the root of the tail near the anus of a goat.

*Case 65.*—A number of puparia of *bezziana* were sent by Veterinary Assistant L. R. Das, Surnanganj, Sylhet, Assam, the maggots having been collected from the vagina of a cow. A retained placenta resulted

in septic infection which evidently attracted a female *bezziana* to oviposit.

*Case 66.*—A number of puparia of *bezziana* were sent by Veterinary Assistant P. A. Parthasarathy, Parlakimedi Veterinary Hospital, Ganjam District, Madras, the larvæ having been collected from a wound on the body of an elephant.

*Case 67.*—A number of preserved larvæ of *bezziana* were sent by Captain H. E. Cross, C.V.D., Camel Specialist, Sohawa, Punjab. The larvæ were collected from a wound on the neck of a bull (yoke gall).

*Case 68.*—A number of preserved larvæ were sent by the same officer from a wound on the foot, just above the fetlock, of a camel.

*Case 69.*—A large number of second and third stage larvæ of *bezziana* in spirit were sent by Veterinary Assistant L. Kumaraswami, Veterinary Hospital, Ongole, collected from the eye of a cow and the cancerous horn of a bullock. Mr. Kumaraswami informs me that cancerous growths of the eye and horn are very common among the well known Ongole breed of Indian cattle, and that these tumours nearly always contain maggots when they become ulcerated.

*Case 70.*—A large number of mature larvæ of *bezziana*, many of which had pupated on the journey, were sent by Staff Veterinary Assistant Babu Satish Chandra Das Gupta, G. B. V. C., Gauhati, Assam, the larvæ having been collected from a wound on the body of a bullock.

*Case 71.*—A number of dead larvæ and puparia of *bezziana* were received from Staff Veterinary Assistant Chatterjee, Silchar, Assam, the larvæ having been collected from a wound on the head of a dog.

*Case 72.*—Four living larvæ and one dead one of *bezziana* were received from Veterinary Assistant Ramaswamy Aiyer, Calicut, collected from an ulcer on the body of a cow.

*Case 73.*—A number of puparia of *bezziana* were sent by Veterinary Assistant G. H. Khan, Bilaspur, the larvæ having been collected from a wound in the vagina of a cow-calf.

*Case 74.*—A number of puparia of *bezziana* were sent by Veterinary Assistant L. R. Das, Surnaganj, Sylhet, Assam, the larvæ having been collected from a wound on the body of a bullock.

*Case 75.*—A number of dead larvæ and many puparia of *bezziana* were received from Veterinary Assistant Moulvi Dewan Faizuddin Ahmad, Gauhati, Assam, the larvæ having been collected from the penis

sheath of a bullock. There was a wound about the middle of the sheath, which was almost destroyed at this point. The animal suffered from retention of urine. Mr. Ahmad says this animal was attacked by *bezziana* several times and thus accounting for the extensive destruction.

*Case 76.*—A number of living larvæ and several puparia of *bezziana* were sent by the same observer collected from a sinus on hoof and pastern of a bullock. A cart had recently passed over the part causing a wound which became infested with the larvæ of *bezziana*.

*Case 77.*—A number of puparia of *bezziana* were received from Babu Narendra Nath Dutta, Senior Veterinary Assistant, Sylhet, the larvæ having been collected from a wound on the body of a bullock.

#### NOTES ON THE DIPTERA WHICH CAUSE MYIASIS IN MAN AND ANIMALS.

Although the invasion of the tissues of man and animals by Dipterous larvæ has long been known, it is only within recent years that most of the flies have been bred out and accurately identified. The difficulty in determining a species from its larva is simply due to our very imperfect knowledge of the distinguishing characters of such larvæ in general, and species in particular. If the fly is not known we are obviously not in a position to study its breeding habits.

These facts could not be better exemplified than in the case of *Chrysomya bezziana*. This calliphorine has in the past been confused with *Chrysomya flavescens*, and *C. megacephala* (Dux) with the result that it has been regarded as a necrophagous species, whose larvæ occasionally cause myiasis. We now know, however, that it never breeds in the decomposing bodies of animals. Further, the cases of myiasis recorded above and in Part I of these notes clearly demonstrate the fact, that the larvæ of *bezziana* may cause rhinal, oral, aural, ocular, cutaneous, and vaginal myiasis. In India much stress has been laid on the condition popularly known as "Peenash" or rhinal myiasis, and it has been erroneously supposed that the larvæ of the fly which cause this condition are only found in the nose. It is now clear that this fly is *bezziana*, and that the mucous membrane of the nose is only one of the tissues selected by the fly in which to breed, and that it will deposit its eggs as readily in any other diseased part. Its larvæ may be found in any form of dermal or cavity myiasis, except gastro-intestinal and urinary myiasis.

It is usual in Text Books of Tropical Medicine to classify the various forms of myiasis according to the tissues or organs invaded, giving the student of the subject the misleading idea that each has its own particular myiasis-producing fly. Although this is to a large extent true in the case of the *Æstridæ*, it is not so for the *Calliphorinae*, *C. bezziana* being a good example of a fly which will take advantage of any diseased tissue in which to deposit its eggs, but its larvæ never cause gastro-intestinal myiasis.

It seems more rational to approach this subject from the standpoint of the flies themselves, rather than the tissues invaded, and we then have the following types of myiasis-producing flies :—

### 1. SPECIFIC MYIASIS-PRODUCING FLIES.

In this group I include *Chrysomya bezziana*, *Wohlfahrtia magnifica*, *Cordylobia anthropophaga*, *C. rodhaini* and all the *Æstridæ*. These flies only breed in living tissues, and their larvæ may cause either dermal, cavity, or gastro-intestinal myiasis. A few notes of what is at present known regarding them may be of interest.

*Chrysomya bezziana* Villeneuve.—As I have already pointed out this Calliphorine will deposit its eggs either on a septic wound or sore, or in a diseased nose, mouth, eye, ear or vaginal orifice. Except for the observations of Rovere mentioned above, there is no evidence that the female will deposit its eggs on the unbroken skin. From 380 to 522 eggs are usually laid, and the larvæ, on hatching out, burrow into the tissues and feed on the fluid which surrounds them ; they are unable to ingest solid matter and certainly do not penetrate bone or cartilage. Structurally they are well adapted to remain fixed in the tissues, the backwardly directed segmental hooks helping to keep them in position ; these hooks are better developed in the first and second stage larvæ than in the third stage. The first stage is a short one lasting from 24 to 36 hours, the second stage from 2 to 3 days, and the mature larva crawls out of the tissues, and dropping on the ground buries itself in the earth to pupate. The adults emerge in about 6 to 8 days according to the temperature. It is not clear what they feed on, but there can be little doubt that they are, to a certain extent, flower feeders. I have not seen any specimens caught in the bazaar meat and sweet shops, and they never enter houses. The female closely resembles the female *C. megacephala*, and it requires a practised eye to separate them

There are several facts connected with the habits of this important Calliphorine which have yet to be worked out. Is the female *bezziana* attracted to fresh wounds in the absence of an offensive discharge, or does it only oviposit on tissues which are septic? Will it lay its eggs on unbroken skin? If undisturbed will the fly lay all its eggs in one batch? It would also be interesting to know the Eastern limit of *bezziana*. Is it found in Malaya, Japan and China? Does it occur in any part of Australia. As far as I am aware, there is no record of the larvæ of any Calliphorine from human tissues in Australia. I will be very glad if any observers who come across cases of myiasis in the e parts will send me the larvæ preserved in spirit, and the fly if they can hatch it out.

*Wohlfahrtia magnifica* Schiner. This species is the specific myiasis-producing fly of South Russia and Egypt, and belongs to the family *Sarcophagidae*, or Flesh flies. It is viviparous in habit, the female according to Portchinsky depositing from 124 to 168 larvæ. The adult flies are flower feeders, and never enter houses. Like its Oriental and Ethiopian ally *bezziana*, *W. magnifica* larviposits on any part of the bodies of man and animals, but the larvæ never gain entrance into the gastro-intestinal and urinary tracts. They are commonly found in the nose, eye, ear and vagina, and in wounds and sores; *Wohlfahrtia magnifica* frequently deposits one or more of its larvæ on the human conjunctiva, and the mature larvæ have been extracted from the eyeball, which may be partially or completely destroyed. The female fly never larviposits in decomposing animal matter. A closely allied species, *Wohlfahrtia meopana* Schiner, on the other hand, normally breeds in decomposing bodies, and its larvæ do not cause myiasis.

*Cordylabot anthropopaga* Blanchard and *C. valharna* God. The larvæ of these two African Calliphorina cause dermal myiasis. Roubaud, Rhodan and others have shewn that their eggs are never laid on the bodies of man and animals, but always in damp earth contaminated with urine or other animal matter. The first stage larva penetrates the skin burrowing obliquely into the subcutaneous tissues, where it develops into the mature larva which later crawls out and pupates in the ground. It is believed that some small animals, probably the dog, in the case of *anthropopaga*, and small deer, in the case of *valharna*, are the normal hosts of the larvæ, and that man is only accidentally affected with the former. There are two close allies of *Cordylabot* in India, particularly in South India, which belong to the genus *Brachyle* var., *Brachyle latro* de Meij. and *B. lateralis* Meq. I have collected many specimens of these

two species at the foot of the Coonoor Ghât at Kallar in damp shady places under bamboos. Practically nothing is known of the life histories of the flies of this genus, and it is more than probable that they are parasitic in small animals in their larval stages. It is hoped more attention will be paid to them in India. Both species never breed in decomposing animal matter.

#### THE CESTRIDÆ.

This family of highly specialised muscids are of peculiar interest, for their larvæ normally live in the tissues and organs of special mammals and their near allies. The adult insects are for the most part incapable of feeding, as their mouth parts are atrophied. The family is at present best divided into five sub-families, the Cephomyiinae, Cuterebrinae, Hypodermiinae, Gasterophilinae and the Cestrinae.

The classification of the Cestridæ is almost entirely based on the characters of the third stage larvæ and those of the adults. I, however, consider that the third stage larvæ does not give the best clue to the relationships of the adults, either to each other or to other Muscidæ. The third stage larvæ of many of the species, which are at present placed in distinct genera on the characters of the adults, are so alike each other, probably due to convergent adaptation, that it is impossible to separate them. The first stage larva, and in some species the second stage, possess many characters which disappear in the third stage, and in many instances this stage is totally unlike the first. It is in the comparative study of the first stage larvæ with those of other related Muscidæ, particularly the Calliphorinae, that we may hope to discover the true relationships of these flies. In any case I cannot agree with Girschner's views that many of the Cestridæ belong to the Tachinidæ, for Girschner based his ideas on the structure of the adults, and did not study the first stage larvæ.

The study of the first stage larvæ of the Calliphorinae and those of a few Cestridæ have convinced me that the latter are, as a whole, closely related to the former, and that when a careful comparative study is made of the first stage larva of such a species as *bezziana* with those of *Cephomyia*, *Cuterebra*, etc., it will be found that they have many characters in common. These characters, however, tend to disappear in the second and third instars. In the case of the Cestridæ it is unfortunate that only a very few first stage larvæ are known, as they are, for obvious reasons, difficult to obtain. It is hoped that in future more attention will be paid

to the study of this stage, and that those who have opportunities of collecting them will do so. I will now give a few notes on the various sub-families.

*Cephenomyiinae*.—These flies are parasitic in their larval stages in the nasal cavities and accessory sinuses of the Cervidae and occasionally in ruminants, and one species in the oesophagus of the African elephant. They are found in Europe, North and South America, and Africa, where they cause cavity myiasis in various species of deer, and the elephant. The flies are viviparous, depositing their first stage larvæ at the openings of the nostrils or mouth, the second and third stage larvæ migrating further in, and often being found in the frontal sinuses, nasopharynx, eustachian tube, and even the trachea. The sub-family contains at least three genera *Cephenomyia*, *Pharyngobolus* and *Pharyngomyia*.

The larvæ of the species of *Cephenomyia* and *Pharyngomyia* are parasitic in the nasal cavities of deer, and the larva of *Pharyngobolus* in the oesophagus of the African elephant. The former crawl out of the nose when ready to pupate, and the latter are passed out in the excreta of the elephant. It is not known whether any species of *Pharyngobolus* parasitise the Indian elephant. I hope those who have opportunities of collecting such larvæ from the Indian elephant will do so, and send them to me preserved in spirit.

Girschner and others place these genera in the sub-family Calliphorinae, but I consider this is premature. The few adults I have had an opportunity of examining, though suggesting affinities with the Calliphorinae do not justify their being classed with them. Ferries has recently described the first stage larvæ of *Cephenomyia americana* and this is, as far as I am aware, the only detailed description of this stage of the flies of this sub-family. The third stage larvæ are very difficult to separate from those of other *Cestrids* which live in the nasal cavities of animals.

*Hypodermatinae*.—This sub-family contains the well known warble flies whose larvæ are found under the skin of cattle, buffaloes, goats, and deer. It has long been a mystery as to how these larvæ reach the subcutaneous tissues of the backs of these animals, until Carpenter and his co-workers in Great Britain, Glaser in Germany, and Hedwen in Canada, worked out the method by which the larvæ reach their final destination. The eggs are laid on hairs on the legs, and the first stage larvæ, on hatching out, penetrate the skin adjacent to the hair, causing a temporary dermal myiasis. They now migrate upwards, to appear

later in the region of the throat as the second instar, in which stage they are very different to the third stage larva. After a time they leave this site and migrate along the muscles of the back, eventually reaching the subcutaneous tissues of the back. They now perforate the skin, and produce the well known warbles, the larva later leaving the skin drops to the ground and pupates.

The warble flies belong to two genera, *Hypoderma* and *Ædamagena*, the former parasites ruminants and deer, while the species belonging to the latter, live in the hides of reindeer.

*Hypoderma* is very common in Northern India, and Captain Cross, C.V.D., Camel Specialist, tells me its larvæ are plentiful in the hides of cattle, buffaloes, and especially goats, in the Punjab. It would be interesting to know whether there is more than one species in India.

*Cuterebrinae*.—This family at present contains a number of genera, such as *Cuterebra*, *Bogeria*, *Pseudogametes*, *Rogenhoferia*, and *Dermatobia*, some of which are doubtfully valid. The larvæ of *Cuterebra*, and the allied genera *Bogeria*, etc., are dermal and subcutaneous parasites of small mammals, such as rabbits, squirrels, rats, chipmunks, mice, etc., while the larvæ of *Dermatobia* are found in the skin and subcutaneous tissues of man and animals. Exactly how the larvæ of *Cuterebra*, etc., get into the subcutaneous tissues is at present not quite clear, but some interesting observations of Parker and Wells, and Hadwen, seem to suggest that the eggs are laid on the hairs of the host, and that, when the animal licks the egg-laden hairs, the larvæ are swallowed, and that later they migrate to the skin; on becoming mature they crawl out and pupate in the ground. Very little is known of the early stages of these species, but as the adult flies readily oviposit in captivity, it should be easy to obtain the first stage larvæ; they apparently live for a long time in the eggs awaiting an opportunity of finding their way into a suitable host.

*Dermatobia hominis* is the well known species whose larvæ are parasites of the skins of animals and occasionally man in Central and South America, and neighbouring Islands. It is only within recent years that the method by which the larvæ find their way into the skin has been discovered. Like *Cordylobia anthropophaga*, the eggs are never laid on the skin of the host, the larva parasitises, but the female fly has the remarkable habit of catching certain species of mosquitoes and flies, and gluing its eggs in small batches on the ventral surfaces of their bodies. When the egg-carrier seeks a host on which to feed, the warmth of the body of the host stimulates the young larvæ to activity,

and one or more, on emerging from the eggs, at once penetrate the skin of the host. It is very probable there is more than one species of *Dermatobia*.

*Gastrophilinae* or *Gasterophilinae*. This sub-family contains at least two genera, *Gasterophilus* and *Gyrostigma*. All the larvæ of the known species of the former are parasites of the stomach and intestines of the Equidae, those of the latter live in the stomachs of different species of Rhinoceros.

The larvæ of at least three species of *Gasterophilus* are known to live in the stomach and duodenum of the horse, *G. intestinalis*, *G. nasalis* and *G. haemorrhoidalis*. The eggs of the former are laid indiscriminately on the hairs of the horse, but more particularly on the hairs on the inner sides of the fore legs; those of *nasalis* are laid on the hairs between the two lower jaws, and those of *haemorrhoidalis* on the hairs of the lower lip. The larvæ of *G. intestinalis* reach the stomach by being swallowed when the animal licks egg-laden hairs, but the larvæ of *nasalis* and *haemorrhoidalis* leave the eggs without the aid of moisture and penetrate the skin causing a temporary dermal myiasis, and later they migrate to the stomach. As far as I am aware *G. intestinalis* var., *bengalensis* is the only species found in India.

The larvæ of several species of *Gyrostigma* are known from different species of Rhinoceros, *G. sumatrensis* from *Rhinoceros sumatrensis* and *Rh. lasiotis*, *G. conjungens* from *Rhinoceros bicornis*, *G. murchiesi* from *Rhinoceros bicornis* and *G. pavesi* from *Rhinoceros bicornis* and *Rh. sinensis cottoni*. Rodhain and Bequaert have given an excellent account of the various stages of the oestrids of this genus, and particularly a description of the first stage larvæ of *G. pavesi*. The eggs of the latter species are laid on the skin of the head, ears and neck of the animal, and it is probable that the first stage larvæ, on hatching out, penetrate the skin causing a temporary dermal myiasis, later pass along the tissues, and eventually reach the stomach; they may, however, reach the stomach by being licked off from the hairs. The mature larvæ are passed out in the excreta of the Rhinoceros. It is not known whether the Indian Rhinoceros is parasitised with the larvæ of a species of *Gyrostigma*.

*The Estrinae*.—Rhodain and Bequaert in their revision of the *Estrinae* include five genera in this sub-family as follows:—

Genus *Cephalopos*.—This genus contains one species, *C. titillator*, the dromedary and camel bot fly. Its larvæ live in the nasopharynx.

and when mature, they crawl down the nasal passage, often causing the animals to sneeze violently, when the larvæ are ejected. This fly, and perhaps a variety, is very common in India wherever its hosts are to be found. It is viviparous in habit, depositing its larvæ at the openings of the nostrils. The first stage larva is not known.

The genus *Rhinæstrus* contains a number of species whose larvæ live in the nasal cavities of hippopotami, pigs, horses and their allies. One species *Rhinæstrus purpureus* which is widely distributed in Southern Europe, North Africa, Asia Minor, and throughout Southern Russia, is a dangerous human pest. It has the habit of depositing a larva on the human conjunctiva, which is soon penetrated, and unless steps are immediately taken to remove it, may cause partial or complete loss of sight. All the species normally breed in the nasal cavities of their hosts, and as far as is known; all are viviparous.

*Genera Kirkiæstrus, Gedelæstia and Œstrus.*—The species which belong to these genera live in their larval stage in the nasal cavities and sinuses of the bones of the skull of the hollow-horned ruminants; most are found in Africa. *Œstrus ovis*, the well known bot fly of sheep, and goats, is, however, widely distributed, yet we do not know much about its life history and habits. In India it is as common in the goat as the sheep. To what extent it injures these animals is not clearly known, but there can be very little doubt that an animal heavily infected with larvæ must suffer in health. It is erroneously supposed that the larvæ enter the brain, but this is not so, the condition known as staggers being due to other causes.

#### THE STOMACH BOTS OF THE ELEPHANT.

Elephants are commonly parasitised by species of cæstrid which belong to the genus *Cobboldia*, the exact position of which is not certain. Most authorities place them in a sub-family the Cobboldiinae. I have recently had an opportunity of examining a large number of almost mature larvæ of *Cobboldia elephantis* removed from the stomach of a calf elephant. A careful examination of caustic potash preparations of these larvæ suggest some affinities with the Sarcophagidæ. The African elephant is commonly parasitised by the larvæ of two species: *C. crisidiformis* and *C. loxodontis* while those of *C. elephantis* are found in the stomach of the Indian elephant. According to Rhodain and Bequaert the female *C. loxodontis* lays its eggs on the tusk of the elephant near the lip, and the larvæ can thus easily find their way into the alimentary

tract. Very little is known of the biology of these interesting flies, and practically nothing, of the species found in the elephant in India.

On making some enquiries regarding the prevalence of the larvae of *C. elephantis* in the Indian elephant, I have been told that they are extremely common and cause considerable mortality among the animals. Older elephants, when heavily infested, soon get out of condition, owing to digestive trouble; and the young animals become anæmic and weak. Recently Mr. Chari, Special Veterinary Inspector, Mount Stuart, sent me a large number of the larvae of *C. elephantis* removed post mortem from the stomach of an elephant calf. In this case, to quote Mr. Chari's words, there were so many larvae in the stomach of the animal, "that there was no room for a pin to pass through the stomach" and it looks as if this animal died as a result of this heavy infestation. It is, therefore, very evident that *C. elephantis* is believed to be a very serious pest, and that many valuable animals are lost through the infestation by the larvae, and the resulting lesions produced in the stomach. If this is the case, and there seems to be no doubt that it is, the study of the life history of this species is urgently called for, in order to discover some means of controlling it, and treating the infested animals.

Lastly there is another interesting œstrid, which is, at present, only known in its larval stage. It is found in the skin of the foot of the African elephant, and is placed in the genus *Neocuterebra*. Bequaert, in an interesting paper, suggests that this œstrid is allied to *Cordylabua* and that it certainly has no affinities with the *Cuterebrinae*.

## 2. SEMI-SPECIFIC MYIASIS-PRODUCING FLIES.

In this group I include all those species, which though normally breeding in decomposing animal and vegetable matter, will occasionally lay their eggs in living tissues attracted either by a foul smelling discharge, soiled wool, or even by fresh blood. The following species belong to this group:—*Cochliomyia macellaria* Fabr., *Cochliomyia vicina* Rob. Desvd., *Chrysomya megacephala* Fabr., *C. marginatus* Wied., *Phormia regina* Mq., *Lucilia argyrocéphala* Macq., *Lucilia sericata* Mq., *Lucilia caesia* Lin.; all the Australian sheep maggot *Calliphoridae*; certain species of *Sarcophaga* such as *S. haemorrhoidalis* Fabr., *S. ruficornis* Fabr., and many others which have not been accurately identified, and *Aphachuta xanthana* Speiser and *A. rufipes* Meigen.

*Cochliomyia macellaria* Fabr., the well known American Screw-worm fly, which can be distinguished from all the old world *Calliphoridae*

by the three dark longitudinal stripes on the thorax, is a serious pest in the Southern States of North America, Central and South America. Its habits and life history have been carefully described by Bishopp, Dunn, and others. It deposits its eggs in much the same situations as *Chrysomya bezziana*, such as the nostrils, vaginal orifice, eyes, ears, mouth and in all sores, cuts and wounds. But unlike its old world ally *bezziana*, it breeds as readily in fresh corpses, in the dead bodies of animals, and to a certain extent in decaying vegetable matter. The fact that it is necrophagous in habit renders control extremely difficult, for in countries like Central and South America, the dead bodies of animals are always available for it to breed in.

According to Dunn the maximum number of eggs laid in a batch would appear to be 287, a much smaller than is laid by *bezziana*. The larvæ mature in about 6 days when they pupate, and the flies emerge six or seven days later. The female flies often cross their wings scissor-like, and then have a very characteristic appearance. They feed on flower juices as well as on decomposing matter.

*Cochliomyia viridula* Robin-Desvd. This new world Calliphorine, though larger, is very like its ally, *macellaria*, in general appearance, and has the three characteristic dark thoracic stripes. Its larvæ are known to cause cutaneous and cavity myiasis in British Guiana, Trinidad, and neighbouring Islands. It normally breeds in decomposing animal matter. It would be interesting to know more about this species, and I hope that those who have opportunities of obtaining its larvæ will send me specimens.

*Chrysomya megacephala* Fabr.—This is the Oriental Blue Bottle, and is very common in all Indian Bazaars, particularly on meat and sweet stalls; it is especially attracted to toddy and, as Fletcher has pointed out, fouls the pots when attached to the trees. It occasionally causes cutaneous myiasis in animals, but, so far, its larvæ have not been found in human tissues. It normally breeds in decomposing animal-matter, particularly in the dead bodies of animals. It is widely distributed in India, Burma, Assam, and Ceylon, and I have seen a specimen from the Australian Region.

*Chrysomya marginalis* Wied.—This is essentially an African species, being distributed from the North to the South; Austen records it from Quetta. I have not seen any specimens from India. Its larvæ occasionally cause cutaneous myiasis, much in the same way, as do the

larvæ of *meigecephala*. It normally breeds in decomposing animal matter.

*Phormia regina* Meigen. — This dark Calliphorine is a well known sheep-maggot fly in parts of North America, particularly California, laying its eggs in soiled wool.

*Lucilia argyricephala* Macq. — According to Roubaud, this Calliphorine is one of the specific myiasis-producing flies of Africa, where its larvæ have been recorded from cases of human and animal myiasis. This species is the common Green Bottle Fly of India, and though its larvæ may occasionally be found in the tissues of animals, it breeds in fresh meat, in the bodies of recently dead animals, and, to a certain extent, in decaying vegetable matter.

*Lucilia sericata* Meigen. — This Calliphorine is the common European sheep-maggot fly, and is a serious pest in Scotland, depositing its eggs in soiled wool. It is widely distributed and is one of the sheep-maggot flies of Australia. I have bred it in England from decaying vegetable matter. Though common in Mesopotamia, I have not seen any specimens from India, where *argyricephala* seems to take its place.

*Lucilia caesar* Lin. This is another European and Australian species, whose larvæ are occasionally found in the tissues of animals. It is, however, mainly necrophagous in habit.

#### THE AUSTRALIAN SHEEP MAGGOT FLIES.

The life histories and habits of the Australian Blow Flies have been very carefully worked out by Mr. W. W. Froggatt, Government Entomologist, New South Wales, who has also described and illustrated all the species. This knowledge has been of immense value to the sheep farming industry in Australia, for several of these Calliphorines deposit their eggs in soiled wool, and their larvæ burrow into the tissues, not only causing the death of a large number of sheep, but the loss of millions of pounds of valuable wool. The following are the important species :

*Anastellorhina augur.*

*Pollenia stygia.*

*Neocalliphora ochracea.*

*Chrysomya curipes.*

*Chrysomya albiceps.*

*Lucilia sericata.*

*Lucilia tasmaniensis.*

Mr. Frogatt points out that *A. augur*, *P. stygia*, and more particularly *C. albiceps*, have only within recent years acquired the habit of blowing sheep wool, and *C. albiceps* has now become the dominant species, and has to a large extent, ousted *A. augur* and *P. stygia*. I have suggested elsewhere in these notes another possible explanation for the decrease in the numbers of the other two species. The third stage larva of *Chrysomyia albiceps*, in India, and Mesopotamia, feeds entirely on other larvæ and the female fly deposits its eggs among the eggs of other Calliphorinae in decomposing bodies. It would be interesting and important to know whether the larvæ of *albiceps* are necrophagous in habit in Australia in the absence of other larvæ? Will the fly lay its eggs in soiled wool, which does not already contain either the eggs of larvæ of other Calliphorinae? If further observations on the third stage larvæ of *albiceps* shew that it does not feed on, and destroy living tissues, and that the fly only oviposits in soiled wool which contains either the eggs or the larvæ of other Calliphorinae, then *Chrysomyia albiceps* must be regarded as a useful species. My observations on the breeding habits of this fly in Mesopotamia rather tend to support this view, for I have pointed out that in that country its larvæ feed on the larvæ of *Musca determinata* and *M. humilis* in heaps of horse manure, which is a most unusual breeding ground for any Calliphorine.

The third stage larvæ of *Chrysomyia villeneuvei* Patton, are even more predaceous than those of *albiceps*, and a few of these will destroy a large number of other larvæ. It seems to me it would be worth considering the introduction of this species into Australia.

#### THE SARCOPHAGIDÆ.

The larvæ of many species of *Sarcophaga* have been recorded from cases of myiasis, the flies being attracted to foul smelling sores on man and animals, but only a very few have been accurately identified. The flies normally breed in decaying animal matter, but it is clear that some species have developed the habit of depositing their larvæ in living tissues. *Sarcophaga ruficornis* Fabr., is believed to be the common myiasis-producing species in India, but this is not certain. I have been told that cutaneous myiasis, due to the larvæ of *Sarcophaga*, is common in South India, particularly on the East Coast, but so far I have only had such larvæ from two cases from Nellore. *Sarcophaga chrysostoma* Wied., is another noted myiasis-producing species in British Guiana

and neighbouring parts. It is hoped more attention will be paid to these flies in the future, and that all such larvæ will be reared to maturity, the adults being carefully pinned, with their puparia especially the males and some of the larvæ preserved in spirit. I have elsewhere pointed out how the larvæ of *Sarcophaga* may be distinguished from those of the Calliphorinæ (see Part VI of these notes).

#### APHIOCHAETA.

One species of *Aphiochaeta*, *A. xanthina* Speiser, is a well known myiasis-producing Phorid, and I have in this journal recorded several cases of myiasis caused by its larvæ; and another species *A. rufipes* Mg., which appears occasionally to cause myiasis in animals in India. *A. xanthina* is a widely distributed species occurring throughout the Tropics. It has been recorded from West Africa, Central, and South America, and British Honduras; it is found throughout the plains of India, Burma, Assam and Ceylon. It breeds in a variety of food stuffs, such as horse manure, decaying meat, dead insects, and in cats' and dogs' dung. *A. rufipes* Mg. is also widely distributed and breeds in similar substances. There can be very little doubt that when the larvæ of these flies are better known they will be found in many more cases.

#### 3. ACCIDENTAL MYIASIS-PRODUCING FLIES.

In this group I include all those flies whose eggs or larvæ accidentally find their way into the alimentary tract. In the vast majority of cases they are ingested in food or drink, and it is surprising to find how careless even civilised people are in this respect, when we recall the different kinds of larvæ which have been found in cases of intestinal myiasis. For example the drinking of ditch water containing the larvæ of several species of *Eristales*, and *Helophorus pendulus*; the eating of cow dung containing the eggs or larvæ of *Phlebotomomyia crassirostris*; eating fly blown meat containing the eggs or larvæ of the Sarcophagidæ, the *Calliphorinæ* or *Aphiochaeta*; eating uncooked vegetables containing the larvæ of different species of the Anthomyidæ, especially *Fannia canicularis*; eating fruit containing the eggs or larvæ of *Drosophila*, etc.

Most of the eggs of these flies hatch out when ingested, and the larvæ feeding on the contents of the intestines are able to reach maturity. And in the case of *Aphiochaeta xanthina* the larvæ

pupate in the alimentary tract and the flies may even hatch out in the intestines.

In considering the flies which cause intestinal myiasis, it should be clearly remembered that they are not specific myiasis-producing species in the sense that they lay their eggs in man's food in order to reach his intestine; his various food stuffs are only some of those these insects select as food for their larvæ.

#### THE PREVENTION AND CONTROL OF THE MYIASIS-PRODUCING DIPTERA OF INDIA.

*Chrysomya bezziana* is the most important myiasis-producing Calliphorine of India, and the fact that it only breeds in living tissues of man and animals makes the task of control a most hopeful one. On the contrary the control of the Screw-Worm Fly of America is a hopeless task as this fly regularly breeds in decaying animal and vegetable matter.

If all Medical and Veterinary Officers in India were to make a point of destroying all the larvæ of *bezziana* found in cases of myiasis, its numbers would be very materially reduced. But the most important factor in its control will be the education of the masses in India. I would, therefore, strongly recommend the various Health and Welfare Associations in India, to include the subject of myiasis in their curricula of lectures. People should be taught the great suffering and loss *bezziana* annually causes in India, that it only breeds in living tissues, that its very existence in India depends on the carelessness of people in not protecting themselves, and their animals, from its attacks, and that it will only be by united effort that this pest can be reduced and perhaps exterminated.

The efficient protection of all wounds, sores, etc., in any part of the human body is of the first importance, and when invaded, all larvæ should be destroyed and not allowed to crawl away and pupate. A reference to the notes of the cases of myiasis I have recorded, clearly shew that *bezziana* will deposit its eggs even in the most trivial sore, and that unless the case is attended to at once, considerable destruction of tissues will follow. The most serious results, however, follow the invasion of the nose and accessory sinuses by the larvæ of *bezziana*. If the fly has succeeded in laying a large number of eggs hundreds of larvæ soon penetrate high up into the sinuses and are then extremely difficult to dislodge. In the same way the internal ear may be

invaded and destroyed. A swollen face and nose in an otherwise healthy person should at once suggest myiasis due to the larvæ of this fly, and vigorous treatment with douches of chloroform water should be given.

It is most important to protect helpless children, and aged and insane people from becoming parasitised, and people with an offensive discharge from either the nose or ears, should be taught to keep a plug soaked in some simple antiseptic in these natural openings. A nose bleed should be attended to at once. I am told on good authority that cases of elephantiasis in beggars on the West Coast of India are a fruitful source of *bezziana*, the large ulcers which form on their legs attracting the female fly to oviposit; these people should be made to protect their sores from infection.

But there can be very little doubt that *bezziana* finds its most extensive breeding grounds in the bodies of the larger animals. Wounds on animals are so commonly neglected in India, and are often difficult to protect, and even in Veterinary Hospitals they are apt to become blown. Neglected sores on the backs of ponies and donkeys is a common sight in India, and I have no doubt they annually produce large numbers of *bezziana*. Animals which have recently given birth to young often have vaginal wounds which *bezziana* readily takes advantage of. In America, a protective dressing of equal parts of beeswax, fish oil and carbon tetrachloride worked up with sufficient vaselene to give it the necessary consistency is largely used to protect sores, wounds, etc., on animals, from becoming infected with the larvæ of the Screw-Worm Fly; this dressing might with advantage be used in all Veterinary Hospitals in India, especially after the rains, when *bezziana* is most active. In treating infected wounds, etc., it is necessary to remember that the larvæ should be induced to leave the tissues and that they should not be killed *in situ*. It is, therefore, important to first spray the infected part with a little glycerine to cause the maggot's to become active, and then to spray them with chloroform water.

These remarks apply equally well in the case of the semi-specific myiasis producing flies. It is a fortunate thing for the people of India that the purely necrophagous species such as *Chrysomya megacephala*, *Lucilia argyrocepala*, *Aphrocheta rufithorax* and *Sarcophaga* Sp. are only semi-specific myiasis producing flies, and that they normally breed in decaying animal matter, and only occasionally deposit their eggs or larvæ in living tissues.

I have said enough to shew that there is a great opportunity of teaching the masses of India how to protect themselves and their animals from the attacks of *bezziana*, and that, if some of my suggestions are carried out, myiasis, from being an every-day sight, will become a rarity.

In concluding these notes on the Indian Calliphorinae, I would like to say that I will always be glad to help Medical and Veterinary Officers in identifying any of these flies and their larvæ, and further, I will be glad of any material bearing on this subject, particularly any species of myiasis-producing *Sarcophaga* and any of the larvæ and adults of the *Æstridae*. Very little is known of the *Æstridae* parasitic in wild animals particularly deer and antelopes in India, and much material might be collected by Medical officers and others when on Shikar trips. Specimens should be sent to me at the Zoological Department, Edinburgh University.

I wish also to take this opportunity of thanking the Governing Body of the Indian Research Fund Association for their generous financial help which alone has made this enquiry possible, and all those Medical and Veterinary officers who have collected and sent me the larvæ of the myiasis-producing flies of India.

# NOTES ON SOME INDIAN APHIOCHAETAE.

APHIOCHAETA XANTHINA SPEISER. (REPICTA SCHMITZ  
CIRCUMSETOSA DE MEJERE : FERRUGINEA  
BRUNETTI, WHOSE LARVÆ CAUSE  
CUTANEOUS AND INTESTINAL  
MYIASIS IN MAN AND  
ANIMALS,  
AND  
APHIOCHAETA RUFIPES MEIGEN, WHOSE LARVÆ  
OCCASIONALLY CAUSE CUTANEOUS MYIASIS  
IN ANIMALS.

BY

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ILLUSTRATED

BY

EDITH M. PATTON.

(Received for publication, January 13, 1921.)

## INTRODUCTION.

THE flies which form the subject of this paper belong to the family  
Pseudocypselidae, a small group of highly specialised Diptera, with a characteristic  
black-backed appearance and rapid jerky movements by which they can  
always be recognised. Two important characters which are common to  
all the species should, however, be noted. The antenna consists of a large,  
somewhat spherical, third joint which obscures the basal ones, and has

attached to its dorsum in some species, and to its apex in others, a long feathery bristle, the arista. The wings are usually large, the costal vein ending near, or at the middle of, the wing; the first and third long veins are strongly developed, and the latter may be forked at its apex at the end of the costal vein. Four indistinct veins run obliquely across the wing.

Though most species breed in decaying animal and vegetable matter, in birds' nests, and in fungi, some have very remarkable life histories. The larva of one species is known to live in the head of an ant, another lives curled up round the neck of the larva of an ant as a commensal, being fed with the food of the ant larva; some breed in bees and wasps' nests, and one species is known to parasitise the eggs of a spider. But the most remarkable Phorid, a wingless species, *Termitoxenia heimi* Wasm., lives in the nests of various species of Termites and is totally unlike a fly.

The family Phoridae is placed by some authorities at the end of the Orthorapha, and by others in the Cyclorrhapha. There can be very little doubt that the study of their early stages will throw considerable light on their exact position. The fact that the larva of *Aphiochaeta xanthina* possesses serrated mandibles seems to me to suggest relationships with the *Orthorapha*.

During the course of my enquiry into the myiasis-producing flies in India I have had the larvæ of two species of *Aphiochaeta* sent me along with those of *Chrysomyia bezziana*. One of these, *A. xanthina*, is extremely common all over the plains of India, and its larvæ are known to cause myiasis in man and animals; yet it is surprising how few cases have been recorded in India. This is perhaps due to the fact that its larvæ are very small and readily escape detection, and that the adult fly is not known to most Medical and Veterinary officers. I propose therefore describing and illustrating it, as well as its early stages, and also another species, *A. rufipes*, whose larvæ occasionally cause myiasis in animals. The following notes relate to the cases of myiasis from which I have had the larvæ of *Aphiochaeta* sent me.

*Case 1.*—About 20 larvæ of *xanthina* were sent by Rao Bahadur W. V. Kane, L. M. & S., together with larvæ of *Chrysomyia bezziana* collected from the sloughing wound following a perineal abscess. (See Some Notes on Indian Calliphorinæ, Part 7, case 37.)

*Case 2.*—A number of second and third stage larvæ of *xanthina* were sent by Sub-Assistant Surgeon Boishab Charan Salen, Phulbani, Orissa,

1880

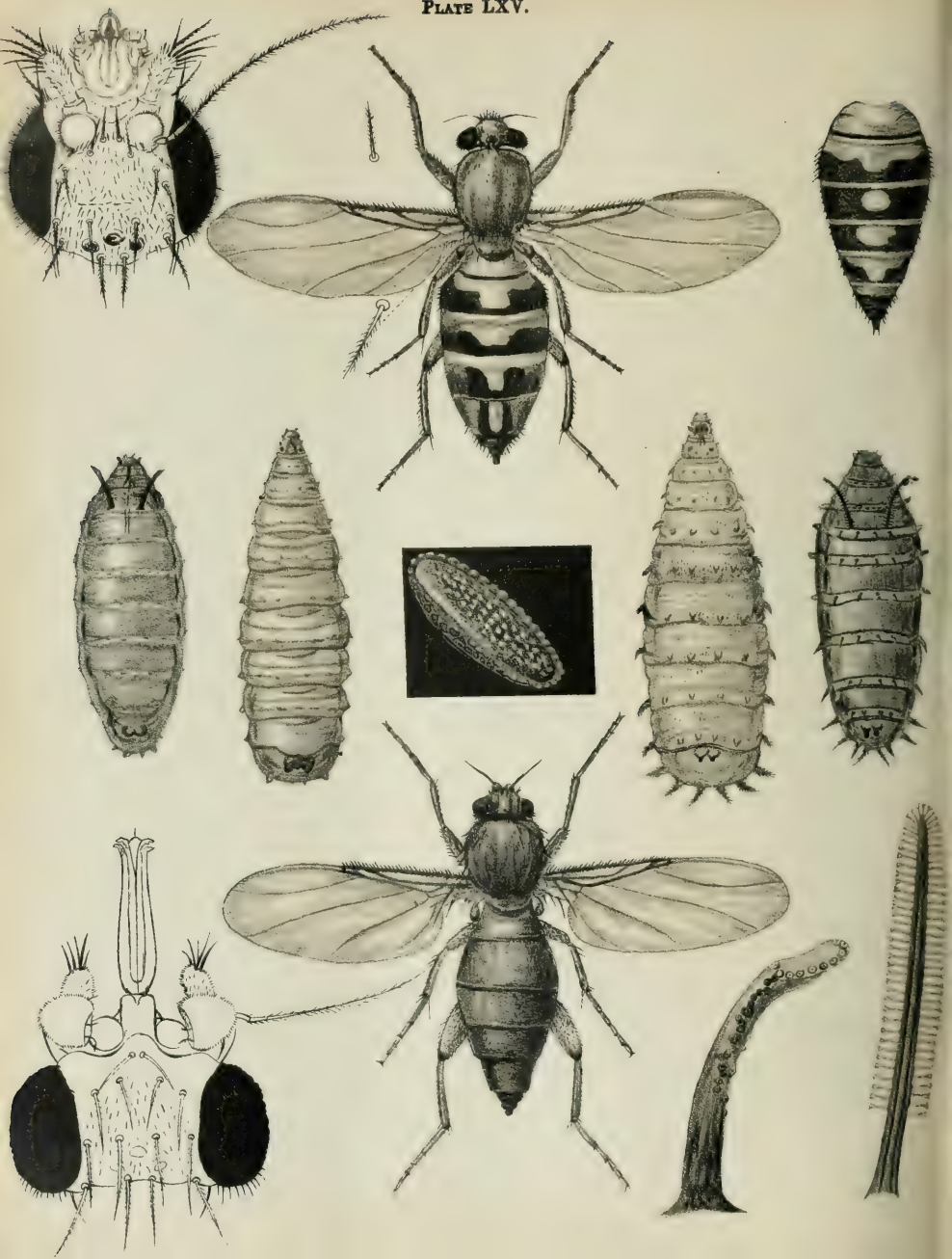


Fig. 3. Head of *Xanthina* ♂, about 60x

Fig. 6. *Xanthina* ♂, 10x

Fig. 7. Abdomen of *Xanthina* ♂, 10x

Fig. 8. Pupa of *Xanthina* ♂, 10x

Fig. 9. Mammotarsus of *Xanthina* ♂, 10x

Fig. 1. Head of *Xanthina* ♂, 10x

Fig. 4. Mature larva of *Xanthina* ♂, 10x

Fig. 5. Pupa of *Xanthina* ♂, 10x

Fig. 11. A pupa of *Xanthina* ♂, 10x

Fig. 12. Head of *Xanthina* ♂, about 60x

Fig. 4. Anterior spiracle of *Xanthina* ♂, 10x

Fig. 10. Anterior spiracle of *Xanthina* ♂, 10x

Fig. 5. Head  
of ♀ xanthina  
× about 60.

Fig. 7. Abdomen  
of ♂ xanthina  
× 16.

Fig. 6. A xanthina ♀ × 16.

Fig. 3. Puparium  
of xanthina  
× 18.

Fig. 2. Mature larva  
of xanthina × 18.

Fig. 1. Egg of xanthina

Fig. 8. Mature  
larva of  
rufipes × 18.

Fig. 9. Puparium  
of rufipes  
× 18.

Fig. 12. A rufipes ♀ × 16.

Fig. 11. Head of ♀ rufipes  
× about 60.

Fig. 10. Anterior  
spiracle of  
rufipes.

Fig. 4. Anterior  
spiracle of  
xanthina.

which were collected together with larvæ of *Chrysomya bezziana* from an ulcer on the scalp of a small boy (See Case 57).

*Case 3.*—A number of mature larvæ of *xanthina* were sent by Veterinary Assistant G. V. Dadhe, Jalgaon, East Khandesh, Bombay, collected from the gangrenous tail of a bullock.

*Case 4.*—A number of second and third stage larvæ of *xanthina* and *rufipes* were sent by Mr. B. B. Joshi, Officer in charge of the Public Veterinary Hospital, Bhamburda, Poona, collected from a wound on the horn of a bull calf.

*Case 5.*—A number of mature larvæ of *xanthina* were sent together with those of *Chrysomya bezziana* by Veterinary Assistant M. R. Tagore, Sevada, East Khandesh, Bombay, collected from a wound on the neck of a bullock.

*Case 6.*—A number of third stage larvæ of *rufipes* were sent by Mr. B. B. Joshi, Bhamburda, Poona, collected from a wound on the tail of a bullock.

#### APHIOCHAETA XANTHINA SPEISER.

##### EARLY STAGES.

The eggs of *xanthina* (Plate LXV, fig. 1) measures .02" in length, and is boat-shaped, and of a silvery white colour. A narrow scalloped frill extends around the egg rather nearer the lower than the upper surface. The upper surface is covered with about 16 rows of about 60 snow-white, short, recumbent spines. The ventral surface is ornamented with regular hexagonal markings. The eggs hatch in 28–36 hours.

A male and a female were placed together in each of seven small corked glass specimen tubes and were given a freshly killed larva of *Lucilia crassa* to feed on. Copulation was observed to take place on the second day after the flies had hatched; the act was of short duration. Fresh larvæ were given as soon as the previous ones had died, and this was accomplished by transferring the pair to another tube in which the larva was crushed. All the males died during the first seven days of the experiment. In each case egg laying began on the third day after copulation and about seven days after hatching. The eggs were laid by the females in batches of from 3 to 80 in number, they were scattered about the tube—sometimes laid on the crushed larvæ, and very often in crevices in the cork. The shortest interval between each batch was about two days, and the longest six days. Seven to eight batches were

laid. As the females were transferred daily to fresh tubes, it was possible to count every egg which was laid. The following gives the counts of the eggs laid by the seven females:—

No. 1. Lived 27 days and laid 218 eggs.

No. 2. Lived 31 days and laid 313 eggs.

No. 3. Lived 31 days and laid 334 eggs.

No. 4. Lived 26 days and laid 344 eggs.

No. 5. Escaped on the 22nd day having laid 142 eggs.

No. 6. Lived 30 days and laid 289 eggs.

No. 7. Lived 30 days and laid 289 eggs.

The average maximum room temperature at the time was 68° F and the minimum 65·7° F, the humidity 84.

The mature larva (Plate LXV, Fig. 2) measures 1/6" in length and is of a dirty white colour. The head segment is small and pointed, the small conical-shaped antennæ projecting from the sides; each consists of a broad, basal, cone-shaped segment, to which is joined two smaller segments. The cephalopharynx is provided with two well-developed mandibles in place of the usual hooks and each is armed with about ten, strong chitinous teeth. The prothorax is short and broad, and has a fleshy pointed process at its anterior and lateral aspect. The anterior spiracles are small, brown, chitinous knobs which project from the sides below and to the outer sides of the fleshy processes. The remaining segments gradually broaden out, the larva being broadest at the posterior end. Each segment is armed with a fleshy process at its upper end and a pair or more on the dorsal surface on each side of the middle line. These processes gradually increase in length, and are longest on the eighth segment. The anterior ends of the segments are covered with a few rows of microscopic spines. The posterior spiracles are situated on the dorsal surface of the eighth abdominal segment; each ends in a brown chitinous hump, the spiracular opening consisting of a narrow slit at the apex. There are two long fleshy processes at the sides of the last segment, and a smaller pair on each side of the middle line.

The mature larva when about to pupate ceases to move and assumes a humped appearance, slowly becoming darker. About the sixth to the eighth day, the anterior respiratory processes begin to project through the skin at the sides of the metathoracic segment. If they are carefully watched it will often be noted that one appears before the other. When they are completely extruded they form long, divergent, rod-like,

chitinous processes, the basal half dark brown and the apical lighter. The end of each is scooped out, the concave side directed downwards and inwards, the openings of the spiracles being situated all along the margin of the hollow and on the outer and lower sides of the shaft. When examined with a high power it will be seen that the minute protuberances are in reality openings of the spiracles (Plate LXV, Fig. 4). The puparium (Plate LXV, Fig. 3) is a little shorter than the mature larva.

The first larval stage lasted about one to one and a half days, the second three to five days, and the third stage from six to eight days. The mature larva took from four to six days to pupate, and the flies hatched out in from seven to nine days. The whole cycle, from the egg to the adult, occupied from 21 to 27 days. The fly, when about to emerge pushes off the upper surface of the puparium which splits along the middle line up to the end of the first abdominal segment.

#### *The Adults.*

Head broad (Plate LXV, Fig. 5), yellowish brown, eyes black, widely separated and covered with minute hairs. Front, broad covered with small bristles: ocellar triangle well marked, especially in the male; ocelli large and situated close together. Two large reclinate bristles close to the posterior ocelli, and two a little distance in front of the ocellar triangle on each side of the middle line. A row of four reclinate bristles running along the inner borders of the eyes and forming a curve, the apical one on the vertex just inside the upper angles of the eyes. A row of four proclinate bristles arranged in the shape of the letter U at the lower end of the front. Two large bristles on cheeks. Antennae short, the basal segments obscured by the globular third segment, the arista (Plate LXV, Fig. 5) projects outwards from the dorsum of the third segment and consists of two short basal segments and a long feathery apical one. Palpi elongated and club-shaped, armed at their apices with five long bristles, four sub-dorsal and one ventral; all bristles of head minutely feathered. The front of the male is slightly narrower than that of the female.

Thorax yellowish brown (Plate LXV, Fig. 6), usually lighter towards the anterior end and often with a faint indication of a dark central stripe, and one on each side; surface of thorax covered with hairs. Pleurae high yellow. Bristles arranged as follows. A pair of bristles at the base of the thorax on each side of the middle line

five bristles along the margin of the thorax and four bristles on the scutellum, the larger pair crossed.

Abdomen, female (Plate LXV, Fig. 6), yellowish brown, sometimes quite light, but usually dark brown. A dark band extending along lower border of apparent second segment, with a break in the middle in the shape of the letter U. Band on third segment somewhat similar, but the break is wider. The band on the fourth segment extends to the lower border of the segment, and the arms of the U nearly reach the upper border, the remainder of the segment much lighter. All the other segments are in most specimens black. These markings are very variable; in some specimens they are much more developed, while in others they are much reduced. In the male abdomen (Plate LXV, Fig. 7) the dark markings are nearly always much more extensive, often only leaving white dots or stripes on the dorsal surface. These appearances are well shewn in figures 6 and 7 on the Plate LXV; they represent the markings on a large number of bred specimens.

External genitalia of male is very complicated and I do not propose attempting to describe it here; the two stiff bristles at the apex of the finger-like process, which appear to be the homologue of the superior clasper, are very characteristic of this species.

Legs pale yellow and covered with numerous hairs and bristles. Coxæ with stiff black bristles; fore femora with several stout bristles at the apex, and tibiæ with a row of short bristles on the front aspect. Mid tibiæ with a very long, stout, black bristle at its apex, and another shorter one at the apical end of the first tarsal segment. Hind femora with a characteristic dark spot at the apical end, and one moderately stout bristle at the apex of the tibiæ, in addition to several smaller ones.

Wings, whitish yellow with two short rows of short, stout bristle along the upper border of costa extending to the bifurcation of the third vein. Five long feathered bristles in a row along the inner margin of the wing in front of the alula in the female and four in the male; this number is characteristic of this species. Venation of wings well shewn in the drawing of the female.

*Aphiochaeta xanthina* is perhaps the most widely distributed species of the genus, having been recorded from most parts of the tropical and subtropical regions of the world, and its larvæ are known to cause myiasis in man and animals. I have bred this species from horse dung, cat and

dog dung, stale and decaying meat and in the dead bodies of insects. It is evidently attracted to foul smelling discharge from sores, etc., in which the female will readily deposit its eggs. Its eggs and larvæ probably gain entrance to the human alimentary tract in food and particularly in stale meat. I have given a large number of eggs and larvæ on pieces of banana to monkeys, but unfortunately, owing to the difficulty of detecting the larvæ in the food, faeces, etc., in the pan below the cages, I have not been able to find any of the stages, and have not been able to discover whether the fly can complete its life-history in the alimentary tract of the monkey. These experiments would be worth repeating.

*Aphiochaeta xanthina* can be bred indefinitely in tubes in the way described above, and the larvæ are extremely hardy and can resist long immersion in various fluids, including alcohol. It is interesting to note that instead of the usual hooks the larva is armed with serrated mandibles not unlike those of the larva of some Nematocera.

### APHIOCHAETA RUFIPES.

#### EARLY STAGES

As I have not bred this species I have not had an opportunity of examining its egg, first and second stage larvæ. The adults were bred out of a number of third stage larvæ sent me from the two cases of myiasis recorded above.

The mature larva (Plate LXV, Fig. 8) measures about  $\frac{1}{8}$ " in length and is of a yellowish white colour. It is very similar in general appearance to the larva of *xanthina*, and this is well shown in the two drawings. It, however, has the ordinary mouth hooks. The prothorax of the larva of *rufipes* is, however, armed with a small half moon-shaped plate of chitin on its dorsal surface, the convex side of the plate being directed towards the head; the anterior spiracles project from the sides of the segment, their apices terminated of small chitinous knobs. On the dorsal surface of the mesothorax there are four small chitinous plates, the outer pair being the largest and almost circular in shape, the median pair are smaller and more or less elongated. On the ventral side of the segment there is a small rectangular plate of chitin in the mid ventral line with two small lobes at its anterior end. All the body segments are provided with six pairs of hairy fleshy processes, which increase in length from before

backwards; one on each side of the outer edge of the segment and a pair on each side of the middle line of the dorsal surface. The posterior spiracles are situated close together on the dorsal surface of the upper edge of the eighth abdominal segment, each consists of a small chitinous hump with the spiracular knob projecting from its apex. The processes at the sides of the segments are long and covered with hairs. The whole surface of the body of the larva is minutely pilose.

The puparium (Plate LXV, Fig. 9) is in every way similar to the mature larva. The anterior spiracles (Plate LXV, Fig. 10), however, instead of being simple rod-like structures with the spiracles opening at the apex as in the case of the puparium of *xanthina*, have attached to them a delicate membrane which gives support to the minute tracheal tubes running out from the main trunk in the rod and ending in minute openings at the margin of the membrane. This type of spiracles is characteristic of the puparia of a number of species of *Aphiochaeta* I have had the opportunity of examining.

#### *The Adults.*

Head (Plate LXV, Fig. 11) somewhat narrower than that of *xanthina*; eyes black, widely separated, and only sparsely covered with hairs. Front broad with some small bristles; two large reclinate ocellar bristles and two below and to the outer sides of the front ocellus; three large bristles close to the eyes and four arranged in pairs at the lower end of the front, the lower pair attached near each other. Third joint of antenna large and conical in shape; arista arising from near the apex and consisting of two small joints and a long sparsely terminal one. Palpi broad and elongated with five bristles at the apical end.

Thorax (Plate LXV, Fig. 12), dark brown, often with a reddish tinge, and in dried specimens appears almost black, covered with minute hairs. Bristles arranged as follows:—

A pair of bristles at the side of the base of the thorax; a long scutellar bristle and a smaller one at each side near the base; five bristles situated along the margin of the thorax.

Abdomen (Plate LXV, Fig. 12), dark brown, often blackish in dried specimens; ventral surface, light yellow. Legs, light yellow; fore tibiae with a row of five short bristles on the outer aspect; mid tibiae with two long, stout bristles on the outer side just below the apex on the inner side. Hind femora short and stout; tibiae with two large

bristles on the inner side. Wings with a double row of short bristles on the costa extending up to the bifurcation of the third vein; a small bristle on the third vein near its basal end, and a long stout one on the basal end of the second vein. A single feathered bristle at the inner margin of the wing in both sexes.

As far as I am able to ascertain this species is Meigen's *rustipes*; its larvæ have, I believe, been recorded from myiasis in man. I wish to thank Mr. Brunetti for giving me the correct synonymy of *A. canthina*.

# BACTERIOLOGICAL AND LABORATORY TECHNIQUE.

## Section V.

BY

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[Received for publication, June 21, 1921.]

### C4 CULTURE METHODS.

#### C4.1 AEROBIC<sup>1</sup> CULTURE.

**Notes.**—<sup>1</sup>Culture under conditions permitting of free access of air. The methods given under this heading are general and are equally applicable to anaerobic culture. Many of the activities of bacteria, such as fermentation, etc., and which are described under a separate heading, might be described as culture methods.

#### C4.11 SOWING.

**C4.111 FLUID MEDIUM.**—(1) Grip firmly the lower ends of seed T.T. and culture T.T. between fingers and thumb. (2) Pass the wool plug and the mouth of each T.T. through the Bunsen flame. (3) Extinguish the lighted wool. (4) Remove the plugs and place on the laboratory bench. (5) Take up seed material from the seed culture on a platinum loop<sup>1</sup> and transfer it rapidly to the culture medium in which it is to be sown. (6) Distribute the seed in the medium. (7) Sterilize the platinum loop in the Bunsen flame. (8) Pick up each wool plug in turn with sterilized forceps and pass through the Bunsen flame. (9) Extinguish the lighted wool. (10) Replace the wool plugs in their T.T. (11) Pass the wool plug and the mouth of each T.T. once more through the Bunsen flame and extinguish the lighted wool. (12) Write details of culture on the freshly sown T.T. with a glass pencil. (13) Incubate.

**Notes.** —<sup>1</sup>Other instruments for sowing are straight platinum wire, thin glass rod with slightly turned up end, badger hair brush, capillary pipette, and syringe.

**C4-112 SURFACE<sup>1</sup> MEDIUM, SLOPE.**—(1) Place the end of a platinum loop or other instrument containing seed material on the lowest part of the surface of the medium slope<sup>1</sup>. (2) Draw it upwards along the length of the surface in straight or closely zig-zag line.

**Notes.**—If the surface is dry, moisture can be made to exude upon it by heating the medium in a free flame without melting it. The same principle of distribution of seed holds good for plate surface sowing. The medium may be nutrient agar, gelatin coagulated serum, egg, etc.

**C4-113 SURFACE MEDIUM, PLATE.**—(1) Melt the medium<sup>1</sup> to be used by keeping it at boiling water temperature. (2) Allow to cool to between 42 and 45C. (3) Pour a sufficient quantity of the melted medium into the under portion of a slightly warmed Petri plate to form a thin continuous layer. (4) Apply the plate cover. (5) Allow the medium to set fast. (6) Sow<sup>2</sup> test material by distributing it over the surface of the congealed medium with platinum needle, platinum loop, right angled glass rod, or other suitable instrument. (7) Incubate the plate in the inverted position.

**Notes.**—The medium may be contained in a flask or T.T. It may be gelatin or nutrient agar. <sup>2</sup>It is an advantage to sow on a dry surface so that spreading of colonies may not take place. The plate may be dried, if necessary, in the incubator or hot air oven before sowing, or, if at the time of sterilization, a strip of filter paper be fastened into the cover of the plate with sealing wax, and with its ends projecting to the outside, the condensation water which forms will be soaked up and evaporate away. To avoid falling of condensation water, if it forms, upon the sown agar surface.

**C4-114 SURFACE SERUM AGAR PLATE.** (1) Melt 3 T.T. of nutrient agar by keeping at boiling water temperature. (2) Allow to cool to between 42 and 45C. (3) Keep 3 T.T. containing sterile blood serum in a water bath at 42C. (4) Pour the contents of each of the serum T.T. into one of the T.T. containing melted agar. (5) Mix by rotating the T.T. between the palms of the hands. (6) Pour, while still fluid, into a Petri plate. (7) Prepare two similar plates with the remaining serum and agar. (8) Dry the prepared plates at 60C. (9) Apply a small quantity of the test material to the surface of the serum agar of No. 1 plate. (10) Spread the test material over the surface with a sterile right angled glass rod. (11) Sow the 2nd<sup>1</sup> and 3rd<sup>1</sup> plates with the same glass rod without recharging it or re-sterilizing it.

**Notes.** <sup>1</sup>Discrete colonies, if not obtained on No. 1 plate, will, at all events, be obtained on No. 2 or 3.

**C4-115 SUBSTANCE MEDIUM, PLATE.** (1) Melt the medium to be used by keeping it at boiling water temperature. (2) Allow to cool to between 42 and 45C. (3) Pour a sufficient quantity of the melted

medium into a Petri plate to form a thin continuous layer. (4) Allow to set fast. (5) Pour melted medium, which has been already sown with test material, on to the layer of congealed medium in the plate. (6) Allow to set. (7) Cover this second layer again with a thin layer of melted medium. (8) Apply the plate cover. (9) Allow to set. (10) Incubate<sup>1</sup>.

**Notes.**—<sup>1</sup>Organisms develop into colonies in the substance of the middle layer, and difficulties connected with the spreading action of water of condensation are avoided.

**C4-116 SUBSTANCE MEDIUM, PLATE.**—(1) Melt the medium<sup>1</sup> by keeping it at boiling water temperature. (2) Allow to cool to 42C. (3) Sow material for culture in the substance of the melted medium with platinum loop or needle. (4) Rotate the T.T. between the palms of the hands to mix. (5) Pour the sown medium into a slightly warmed Petri plate. (6) Apply the cover. (7) Invert<sup>2</sup> the plate as soon as the medium has set so as to make the cover the lower of the two portions. (8) Incubate.

**Notes.**—<sup>1</sup>The medium used may be gelatin or nutrient agar. In the case of gelatin the medium can be melted and sown at lower temperatures than in the case of nutrient agar. The medium for this method of plating is usually kept in T.T. in amt. of 10 to 20 c.c. according to the size of plate used. <sup>2</sup>The inversion allows of the condensation water collecting on the cover of the plate instead of on the surface of the medium. If it collected on, or fell on to, the surface of the medium, surface colonies would spread all over the plate and so prevent differentiation. Gelatin plates, used to show liquefaction, are not inverted.

**C4-117 SUBSTANCE MEDIUM, STAB.**—(1) Charge a straight platinum wire with seed material. (2) Pass the charged wire into the culture T.T. held inverted till it touches the upper surface of the medium<sup>1</sup>. (3) Push the charged needle with rapid movement into the medium and withdraw it again.

**Notes.**—<sup>1</sup>The T.T. with its contained medium may be allowed to fall by its own weight on to the charged platinum wire, which is held fixed. Stab culture into the butt of an agar slope may be sometimes usefully combined with surface culture.

**C4-118 SUBSTANCE MEDIUM, SHAKE.**—(1) Melt the medium<sup>1</sup> in its T.T. (2) Allow to cool to 42C or to a lower temperature in the case of gelatin. (3) Sow material for culture in the melted medium with platinum loop or needle. (4) Rotate the T.T. between the palms of the hands to mix. (5) Allow to set. (6) Incubate.

**Notes.**—<sup>1</sup>The medium should be specially transparent to permit of detection of colonies in its substance.

**C4-119 ROLL CULTURE.**—(1) Melt the medium<sup>1</sup> in its T.T. (2) Allow to cool to 42C or to a lower temperature in the case of gelatin.

- (3) Sow material for culture in the melted medium. (4) Roll<sup>2</sup> the T.T. held horizontally until the medium sets upon the surface of the glass.
- (5) Incubate.

**Notes.**—<sup>1</sup>Only a small quantity of medium, about 5 c.c., is required, as the film formed upon the glass should be thin to allow of examination under a low power microscope. If agar is the nutrient medium, it must be of strength 3 or 4 per cent. to adhere satisfactorily to the glass. <sup>2</sup>Rolling in cold water or upon a block of ice accelerates the setting.

#### C4:12 EXAMINATION OF COLONIES.

- C4:121 PLATE CULTURE.**—(1) Remove the cover of the plate. (2) Examine the colonies by naked eye and hand lens under a good light. (3) Note the characters of the colonies. (4) Place the plate on the stage of the microscope. (5) Use a low power objective and eyepiece. (6) Swing out the condenser and narrow down the aperture of the diaphragm. (7) Focus<sup>1</sup> single selected colonies in turn. (8) Note the character of the colonies. (9) Fix on the colonies to be identified. (10) Touch each colony with the point of a sterile platinum needle. (11) Transfer to fresh medium as a pure culture. (12) Subject the culture, after incubation, to further tests for identification.

**Notes.**—<sup>1</sup>Oblique illumination may be required to show up colonies. A beam of strong light after passing through a slit, if obtainable, is very useful for the examination of growths.

- C4:122.**—(1) Examine slope culture colonies by naked eye and hand lens under a good light. (2) Place the T.T.<sup>1</sup> on the stage of the microscope and examine under a low power objective and with a narrowed diaphragm.

**Notes.**—<sup>1</sup>Condensation water on the wall of the T.T. should be removed in the case of slope cultures by gently heating the T.T. Roll, shake, and stab cultures may also be examined microscopically. In the case of the stab culture, if the growth is to be examined microscopically, the stab should be made near the wall of the T.T.

#### C4:13 SEALING.

- C4:131 BLOWPIPE<sup>1</sup>.**—(1) Heat the rim of the T.T. in the blow-pipe flame and fix to it a handle of glass tubing. (2) Heat the T.T. gradually in the blowpipe flame beneath the rim without exerting any pull on it. (3) Produce thickening of glass at this point and narrowing of calibre. (4) Seal with a thickened glass top. (5) Score the glass, to unseal, with a file or glass knife near the top. (6) Apply to the scored point of white hot glass to start a crack. (7) Lead the crack circularly round the tube. (8) Give the top of the T.T. a smart tap and cause

it to fall into antiseptic sol. (9) Subculture by sowing heavily<sup>1</sup> from the growth into fresh medium.

**Notes.**—<sup>1</sup>The best method for certainty of preservation. <sup>2</sup>To increase the chances of obtaining growth.

**C4·132 PARAFFIN.**—(1) Use sterile<sup>1</sup> melted paraffin. (2) Insert the wool plug of the culture whole<sup>2</sup> into the melted paraffin. (3) Insert the paraffin saturated wool into the T.T., held inverted<sup>3</sup> during the process. (4) Push well into the T.T. (5) Fill in the space between plug and rim with melted paraffin and allow it to set. (6) Hold the T.T. inverted to unseal, and melt the paraffin in a free flame. (7) Pull out the plug with forceps, or lever it out with a knife. (8) Subculture by sowing heavily into fresh medium.

**Notes.**—<sup>1</sup>Sterilized by taking up to a high temperature and then allowing to cool down somewhat. <sup>2</sup>If the whole plug be covered with paraffin, there is less opportunity for mould spores to germinate and grow out of the wool plug, and thus less likelihood of contamination of culture. <sup>3</sup>To avoid having melted paraffin run down on to the culture.

**C4·133 VASELIN.**—(1) Use sterile melted vaselin. (2) Proceed as for paraffin.

**C4·134 SEALING WAX.**—(1) Cover the bottom<sup>1</sup> of the wool plug with melted sealing wax. (2) Insert the wool plug well into the T.T. (3) Cover the top of the wool with wax. (4) Unseal in the manner described for paraffin.

**Notes.**—<sup>1</sup>For the same reason as given under paraffin for immersing the whole plug.

**C4·135 RUBBER CAP.**—(1) Remove the wool plug. (2) Apply a sterile rubber cap over the mouth of the T.T.

**C4·136 STOPPERED JAR.**—(1) Use a glass stoppered jar. (2) Wash out the jar with saturated mercury bichloride. (3) Place 2 or 3 folds of filter paper moistened with mercury bichloride sol. at the bottom of the jar. (4) Place a thin layer of vaselin on the stopper. (5) Place the cultures in the jar. (6) Close the jar with the stopper.

#### **C4·14 PRESERVATION CULTURES.**

**C4·141.**—(1) Keep the culture tubes after incubation in the inverted position to prevent the access of moulds and to prevent rapid drying. (2) Flame the wool plugs once weekly even although subculture is not made. (3) Subculture every 2 wk.<sup>1</sup>

**Notes.**—<sup>1</sup>Delicate and especially hæmophilic bacteria will require subculture at intervals of not more than 5d. Such organisms also—gonococcus, meningococcus, pneumococcus, B. influenza, etc.—require to be preserved continuously at incubation temperature. Even for purposes of subculture and examination they should not be kept for long outside the incubator.

**C4-142.**—(1) Seal the culture tube completely either by closing the tube in the blowpipe flame<sup>1</sup>, by applying a sterilized rubber test tube cap,<sup>2</sup> or by dipping the wool plug into melted sterile vaselin or paraffin. (2) Open when required for subculture.

**Notes.**—<sup>1</sup>This is the best method for maximum permanency. Many organisms will remain alive under such conditions for years. Others will require subculture at intervals of 1 month or longer. Certain delicate organisms require to be maintained continuously in the incubator. <sup>2</sup>It is advisable to complete the sealing by dipping the cap into melted paraffin after application.

**C4-143.**—(1) Sow as stab or surface culture in suitable<sup>1</sup> medium. (2) Seal. (3) Keep, if necessary, continuously<sup>2</sup> at 37C.

**Notes.**—<sup>1</sup>Such media are glucose agar, egg medium (**M2-8**, **M4-872**), blood gelatin medium, serum medium (**M2-36**, **M2-61**), aseptic fluid agar, etc., for the more delicate aerobic organisms. Anaerobic organisms are well preserved in alkaline meat medium (**M3-51**). <sup>2</sup>For delicate haemophilic organisms which die quickly when kept at B.T., *e. g.*, gonococcus, meningococcus, pneumococcus, and B. influenza.

**C4-144.**—(1) Use T.T. filled 3-4ths full with freshly boiled medium. (2) Sow<sup>1</sup> by stab (**C4-117**). (3) Incubate.

**Notes.**—<sup>1</sup>For anaerobic culture and preservation of anaerobic organisms add a layer of sterile oil 3 cm. deep after sowing.

**C4-145.**—(1) Preserve in serum heated 30 min. at 60C and protected from oxidation by a layer of paraffin oil. (2) Keep continuously<sup>1</sup> at 37C.

**Notes.**—<sup>1</sup>For delicate haemophilic organisms.

**C4-146.**<sup>1</sup>—(1) Plug the culture T.T. with antiseptic wool. (2) Use an ordinary cork. (3) Bore a hole through a cork of about 2 mm. diameter. (4) Dip the cork in hot liquefied paraffin at high temperature to sterilize it and to coat it. (5) Push the wool plug down the T.T. and insert the cork. (6) Incubate the culture T.T. thus prepared in the inverted<sup>2</sup> position.

**Notes.**—<sup>1</sup>A method which is useful in the incubation of such organisms as B. tuberculosis which require a long time before growth appears. The small hole admits a certain amt. of necessary air. <sup>2</sup>Must air rises and will collect at the top of the T.T. Drying of the medium is thus delayed.

**C4-147.**—(1) Saturate sterile silk threads in the material containing the organisms. (2) Place the threads in small sterile T.T. (3) Dry *in vacuo* over calc. chloride. (4) Keep in the desiccator and in a cool dark place. (5) Sow the threads in nutrient media when required.

**C4-148 UNUSED MEDIA.**—(1) Keep in a closed receptacle to prevent evaporation. (2) Keep wool or blotting paper saturated with oil of cloves in the bottom of the receptacle to prevent the growth

of moulds. (3) Add water to dried up media and resterilize. (4) Resterilize media showing only a slight growth of mould.

**C4·149<sup>1</sup> PERMANENT PREPARATIONS.**—(1) Kill and fix the growth by exposure to formalin vapour 24 hr. (2) Seal the plates, upper and lower portions, with melted paraffin.

**Notes.**—<sup>1</sup>For demonstration purposes. Applicable also to T.T. cultures.

**C4·15 ISOLATION<sup>1</sup> OF ORGANISMS.**

**Notes.**—<sup>1</sup>To obtain pure cultures.

**C4·151 DILUTION.**—(1) Liquefy the contents of 3 solid medium T.T. (2) Sow No. 1 T.T. when sufficiently cool with a small quantity of test material. (3) Rotate the T.T. between the palms of the hand to mix. (4) Transfer 2 loopfuls from No. 1 T.T. mixture to No. 2 T.T. (5) Rotate to mix. (6) Transfer 2 loopfuls from No. 2 T.T. mixture to No. 3 T.T. (7) Rotate to mix. (8) Prepare plates (**C4·116**) from all 3 T.T. (9) Incubate. (10) Examine discrete colonies and select those suitable for subculture.

**C4·152 DILUTION.**—(1) Set up a series of T.T. containing 9·5 c.c. sterile water, salt solution, or bouillon. (2) Add to No. 1 T.T. with a sterile 1 c.c. pipette 0·5 c.c.<sup>1</sup> test suspension. (3) Shake to mix. (4) Sterilize the pipette by aspirating into it and ejecting boiling water. (5) Transfer 0·5 c.c. mixture from No. 1 T.T. mixture to No. 2 T.T. (6) Shake to mix. (7) Transfer 0·5 c.c. from No. 2 T.T. mixture to No. 3 T.T. and so on. (8) Transfer 0·5 c.c. from each T.T. mixture separately or at least from those which are likely to give a satisfactory growth of discrete colonies to T.T. of melted nutrient medium. (9) Pour plates. (10) Incubate. (11) Enumerate and proceed to identify the colonies which have developed. (12) Calculate the number of living bacteria present in the original test suspension.

**Notes.**—<sup>1</sup>The first dilution is thus 1 in 20. A capillary pipette may be used for dilution, using volumes instead of c.c. The degree of dilution and the No. of dilutions may be varied according to circumstances. In this case they are 1·20, 1·400, and 1·8,000.

**C4·153 DILUTION.**—(1) Use 3 T.T. of 1½ in. diam. and containing ¾ in. depth of melted medium<sup>1</sup>. (2) Sow<sup>2</sup> from one T.T. to the other to obtain dilution of the organisms contained in the test suspension. (3) Make roll cultures (**C4·119**) of the inoculated media. (4) Incubate. (5) Examine the colonies which have developed.

**Notes.**—<sup>1</sup>Usually nutrient gelatin. <sup>2</sup>The method may serve for enumeration of organisms if a definite amt. is used in each successive sowing.

**C4'154 DILUTION.**—(1) Place a small quantity of test material on the sloped medium surface<sup>1</sup>. (2) Smear the material by means of a platinum loop<sup>2</sup> in close zig-zag from below upwards. (3) Transfer material adhering to the loop to a 2nd sloped medium surface. (4) Sow as in the case of No. 1 T.T. (5) Carry over test material similarly<sup>3</sup>, without sterilization of the loop, to No. 3 sloped medium surface. (6) Incubate all 3 T.T.

**Notes.**—<sup>1</sup>A similar method may be used for plate surfaces. <sup>2</sup>Or platinum needle.

<sup>3</sup>In this way loss and loss of the test material is carried over with each transference and so the growth of discrete colonies assured.

**C4'155 DILUTION<sup>1</sup>.**—(1) Use sterile capillary pipettes<sup>2</sup> with slightly upturned<sup>3</sup> ends, calibrated to 100 c.mm.<sup>4</sup> capacity. (2) Make suitable dilutions 1-100, 10,000, etc., by adding 100 c.mm. from the test suspension to a T.T. containing 10 c.c.<sup>5</sup> 0.85 S.S.S. or sterile bouillon<sup>6</sup> and from that mixture carrying over the same amt. to a second T.T. containing 10 c.c.<sup>5</sup> of the diluent, and similarly to a 3rd T.T. (3) Shake to mix between each transference. (4) Take up<sup>7</sup> 100 c.mm. from the 1-100 dilution of the test suspension. (5) Distribute this quantity on 3 dry<sup>8</sup> agar T.T. slopes, rubbing each portion as deposited well over each slope. (6) Rest each T.T. without its wool plug on glass tubing or other support, in as nearly a horizontal a position as possible. (7) Allow to dry in this position. (8) Sterilize the mouths of the T.T. and the corresponding wool plugs. (9) Replace the wool plugs in the T.T. (10) Incubate. (11) Proceed in similar fashion for the 1-10,000 and other dilutions. (12) Count the colonies which develop. (13) Calculate<sup>9</sup> from the number of colonies the number<sup>10</sup> of organisms contained in 1 c.c. of the test suspension.

**Notes.**—<sup>1</sup>For enumeration of organisms in suspension by colony count. See also **87-341** and **87-342**. <sup>2</sup>Thirty or more of these calibrated pipettes can be made in an hour. <sup>3</sup>Upturned to avoid scratching the surface of the agar slope. <sup>4</sup>There are many other ways of making the necessary dilution of the test suspension. If it is not convenient to work with capillary pipettes, dilutions may be made in 10-fold steps, by transferring 1 c.c. of suspension from each of a succession of T.T. containing 9 c.c. bouillon to the other. <sup>5</sup>Bouillon is a better diluent than salt sol. Some organisms do rather quickly in salt sol. <sup>6</sup>This amt. should, strictly speaking, be 9.9 c.c. and not 10 c.c. <sup>7</sup>If fresh sterile capillary pipettes are not available, one and the same pipette may be used for all operations by sterilizing at each separate step in boiling water. The boiling water is simply aspirated and ejected several times from the pipette. <sup>8</sup>It is highly important to have the agar slope thoroughly dry. This may be done by placing the T.T. in the incubator on the evening previous to use in an inverted position. Water of condensation is taken up by the wool plug. <sup>9</sup>That is, in the 3 T.T. used, on which to place out 100 c.mm. of one of the dilutions there should read 8, 12, and 10 colonies, respectively making a total of 30 colonies, and the dilution used

was 1-10,000, the calculation would be  $30 \times 10 \times 10,000 = 3,000,000$  organisms per c.c. in the test suspension.

**C4-156 DILUTION TO SINGLE ORGANISM.**—(1) Prepare :- Indian ink 1 : D. W. 9. (2) Sterilize. (3) Allow to stand 2 wk. (4) Pipette off the S. N. F. for use. (5) Place 4 large separate drops of Indian ink, sterilely, on a sterile clean slide contained in a Petri dish. (6) Mix with No. 1 drop a very small quantity of test suspension. (7) Transfer a small loopful of the mixture to No. 2 drop and mix. (8) Transfer a small loopful of the 2nd mixture to No. 3 drop and similarly a small loopful of the 3rd mixture<sup>1</sup> to No. 4 drop. (9) Use a sterilized fine mapping pen. (10) Make a series of dots with the pen on a well-dried gelatin plate, first from the 4th mixture and then from the 3rd mixture. (11) Leave for 30 sec. (12) Cover each dot with a sterile cover glass. (13) Examine each dot with a dry lens<sup>2</sup> and high eye piece. (14) Mark on the under surface of the plate those dots which contain one organism only. (15) Incubate<sup>3</sup> the plates to obtain development of organisms into colonies. (16) Subculture from colonies which have developed from a single organism.

**Notes.**—<sup>1</sup>The process is one of dilution of the original organismal suspension to one showing microscopically discrete and ultimately single organisms. <sup>2</sup>The organisms appear bright against the dark particles of Indian ink. <sup>3</sup>If growth on another medium than gelatin is desired use plates of that medium originally, or transfer the cover glass to the desired medium. The Indian ink and the organism will adhere to the cover glass.

**C4-157 DILUTION TO SINGLE ORGANISM.**—(1) Use a sharp pointed piece of match wood. (2) Sterilize by immersion in formalin. (3) Dry just before use with sterile wool. (4) Make a series of dilutions of the test material. (5) Dip the point of wood into the first of the dilutions. (6) Make momentary contact between the point thus dipped and the centre of a sterile cover glass. (7) Invert the cover glass over a glass cell on a slide. (8) Examine the droplet first with a dry lens and then with an oil immersion lens. (9) Examine in this way a series of cover glasses prepared from each of the dilutions of test material until one is found which apparently contains only a single organism. (10) Remove this cover glass and place it, droplet upwards, in a sterile Petri dish. (11) Deliver a small drop of bouillon on to the droplet containing the single organism. (12) Pipette up bouillon and droplet with a sterile pipette. (13) Sow in a T.T. of bouillon. (14) Incubate.

**C4-158 DILUTION TO SINGLE ORGANISM.**—(1) Prepare a series of sterile coverglasses and a number of sterile clean slides. (2) Pour over the slides under the cover of a Petri dish a thin layer of nutrient

agar. (3) Prepare a series of dilutions of test material. (4) Place the minutest possible droplets from each dilution on the centre of a cover glass. (5) Drop the cover glass, droplet downwards, on to an agar slide. (6) Examine the droplets first with a dry lens and then with an oil immersion lens to find one which contains only a single organism. (7) Remove the immersion oil. (8) Incubate 5 hr. at 37°C. (9) Examine the slides at intervals during this incubation. (10) Make certain by this examination that the colony which is seen forming arises from the original organism and that no other colony, which would indicate the presence of more than one organism, is formed. (11) Replace the slide in the incubator for 15 hr. (12) Sow from the colony which has formed.

**C4-159 DILUTION TO SINGLE ORGANISM.**—(1) Use a "moist" box on the mechanical stage of the microscope. (2) Line the sides of the moist box with wet filter paper and have abundance of water in the bottom of the box. (3) Insure a moist atmosphere also by placing numerous small drops of sterile bouillon on the under surface of the cover around the field of operation. (4) Use a sterile capillary pipette for the operation, the capillary end of which is drawn out into a very fine tip and turned up at right angles. (5) Fill the pipette partly with the bouillon to be used. (6) Adjust the pipette in its special holder<sup>1</sup> horizontally and in such a position that the tip may be brought into focus in the centre of the field. (7) Bring the culture drop containing the test material into the field by means of the mechanical stage. (8) Raise the pipette vertically in its holder until its tip is in contact with the test material. (9) Lower the pipette quickly. (10) Move the mechanical stage until a portion of the cover remote from the test material is brought into view. (11) Raise the pipette at this spot. (12) Blow very gently through the rubber tubing attached to the pipette and so eject a portion of the contents containing the bacteria on the under surface of the cover. (13) Use a new pipette. (14) Proceed to make dilutions of the ejected portion on the under surface of the cover glass until it becomes possible to withdraw a single organism in the pipette. (15) Detach the pipette containing the single organism. (16) Blow out the organism on to an agar plate. (17) Incubate.

**Notes.**—<sup>1</sup>In the absence of a special holder a dissecting microscope with rack and pinion movement can be converted into a holder for these pipettes if the body tube of a second microscope may serve the same purpose. The pipettes may even be manipulated by hand.

**C4'16 ISOLATION OF ORGANISMS.**

**C4'161 SELECTIVE MEDIA**<sup>1</sup>.—(1) Sow on a selective medium<sup>2</sup> of suitable reaction<sup>3</sup>. (2) Incubate. (3) Transfer after a suitable<sup>4</sup> interval<sup>1</sup> to fresh selective medium. (4) Continue the procedure as many times as are necessary. (5) Examine the final culture for purity<sup>5</sup>.

**Notes.**—<sup>1</sup>If 2 organisms are placed under conditions which allow the growth of both but are ever so little more favourable to the one than the other, the favoured one will not only outgrow the other, but will even prevent its development. <sup>2</sup>Such as peptone water for *V. cholerae*, bile or bile salt media for typhoid group organisms, concentrated sugar media for *Tinea* and *Trichophyton*, acid media for *Monilia*, etc. <sup>3</sup>*E.g.*, p H 7·2 to 7·4 for *B. influenzae*. <sup>4</sup>*E.g.*, every 6 hr. *V. cholerae*. <sup>5</sup>Contaminating organisms, for which the conditions are less favourable, are eliminated after a sufficient number of transfers. A combination of various selective methods is very effective.

**C4'162 SELECTIVE MEDIA**<sup>1</sup>.—(1) Grow the expected contaminating organism on the medium first. (2) Remove this growth from the surface (3) Sow the exhausted medium<sup>2</sup> with the mixture<sup>3</sup> of organisms.

**Notes.**—<sup>1</sup>Methods of exhaustion of the medium for contaminating organisms. <sup>2</sup>The medium may be resterilized and refreshed with added peptone before sowing. <sup>3</sup>The undesirable contaminant will be inhibited.

**C4'163 SELECTIVE INCUBATION.**—(1) Incubate at the temperature 42C, 37C, 23C, 16C, etc., optimum for the organism to be isolated.

**C4'164 SELECTIVE REMOVAL BY HEAT**<sup>1</sup>.—(1) Immerse a T.T. containing the test material<sup>2</sup> for a suitable length of time<sup>3</sup> and at a suitable temperature<sup>3</sup> in a water bath to kill off vegetative forms. (2) Sow from the heated material to obtain discrete colonies.

**Notes.**—<sup>1</sup>Especially for isolation of spore bearing organisms. <sup>2</sup>*E.g.*, anthrax blood, hay infusion, infusion of potato which has been incubated for 3d., etc. <sup>3</sup>5 min. at 53C for *B. anthracis*, 5 min. at 100C for *B. subtilis*, 5 min. at 105C for the potato bacillus (*B. vulgaris*), etc.

**C4'165 SELECTIVE REMOVAL BY FILTRATION**<sup>1</sup>.—(1) Filter the test material through a Berkefeld or Chamberland type of candle. (2) Use the filtrate to obtain cultures or to inoculate animals.

**Notes.**—<sup>1</sup>Applicable especially to filtrable viruses.

**C4'166 SELECTIVE REMOVAL BY ANTIFORMIN**<sup>1</sup>.—(1) Cut up the tissue material into small pieces with scissors. (2) Rub up very thoroughly in a mortar with a small amt. of dry sterile quartz sand. (3) Wash the contents of the mortar into a wide T.T. with 15 to 20 c.c. 0·85 S.S.S. (4) Allow the sand to sediment for a few min. (5) Pipette

off the supernatant suspension. (6) Mix thoroughly with an equal vol. of 15 per cent. antiformin. (7) Stir continuously 5 min. (8) Centrifugalize at high speed. (9) Discard the S.N.F. (10) Shake up the sediment with 0.85 S.S.S. (11) Centrifugalize. (12) Repeat the procedure of shaking up and centrifugalizing 3 times in all. (13) Use the sediment from the final centrifugalization for making cultures, or after suspension in 0.85 S.S.S. for inoculation of G. P.

**Notes.**—<sup>1</sup>Applicable especially to tuberculous material. See also C4.451. The antiformin acts upon the other contaminating bacteria before it acts upon the tubercle bacillus. Other substances used in much the same way are sod. hydroxide, ammonia, peroxide of hydrogen and pancreatin. Antiformin is a mixture of equal parts of liquor sodæ chlorinate (B.P.) and 15 per cent. sod. hydroxid.

**C4.167 SELECTIVE REMOVAL BY ALCOHOL.**—(1) Treat fragments of test material<sup>1</sup> with abs. alc. 3 min. (2) Allow the fragments to dry. (3) Sow separate fragments into a number of glucose bouillon T.T. and on maltose agar medium. (4) Grow at R.T. (5) Examine for mycelial growth<sup>2</sup>. (6) Subculture as soon as growth is visible.

**Notes.**—<sup>1</sup>Applicable especially to fungoid affections of skin and hair. <sup>2</sup>E.g., affected hairs or squames. <sup>3</sup>If contaminating growth appears, sterilize the medium and add to it a little more glucose or maltose and a fragment of calc. carbonate. The medium will be exhausted (C4.162) for the contaminating organism and should be resown with test material.

**C4.168 SELECTIVE REMOVAL BY KAOLIN.**—(1) Make a fine suspension of test material<sup>1</sup> in 5 c.c. 0.85 S.S.S. (2) Add 0.35 gm. kaolin<sup>2</sup>. (3) Shake 1 min. (4) Allow the precipitate to settle. (5) Shake again. (6) Filter through sterile filter paper. (7) Sow 15 loops on Conradi Drigalski medium (M3.752, M3.753) or Endo agar (M3.412 to M3.414).

**Notes.**—<sup>1</sup>The method is applied especially to the isolation of *B. typhosus* in faeces. <sup>2</sup>Kaolin added to a mixture of typhoid and colon bacilli absorbs the typhoid bacilli more strongly than the latter.

**C4.169 SELECTIVE REMOVAL BY ANIMAL INOCULATION.**—(1) Inoculate test material<sup>1</sup> by subcutaneous, intraperitoneal, or other suitable route into a suitable animal<sup>2</sup>. (2) Kill the animal after a suitable interval and examine the blood and organs for the organism required. (3) Sow media with suitable material<sup>3</sup> taken at a distance from the site of inoculation.

**Notes.**—<sup>1</sup>Such as blood stained sputum for pneumococcus, independent sputum for *B. tuberculosis*, blood or spleen tissue for *B. anthracis*, gangrenous tissue in gas gangrene, garden soil for spore bearing antraxes, etc. <sup>2</sup>Prefer a mouse or young G. P. for pneumococcus, with isolation from heart blood or internal organs. G. P. for *B.*

tuberculosis with isolation from glands or internal organs; G. P. for *B. mallei* with isolation from glands or testicle; G. P. for *B. anthracis* with isolation from heart blood, or internal organs, etc.

### **C4·17 OBSERVATION OF GROWTH.**

**C4·171 HANGING DROP.**—(1) Place a drop of sterile bouillon on a sterile cover slip. (2) Add a small<sup>1</sup> quantity of the test organism to the bouillon<sup>2</sup>. (3) Place the coverslip, bouillon downwards, over the depression of a sterile hollow slide. (4) Lute the edge of the coverslip with vaselin. (5) Incubate. (6) Examine at intervals on a warm stage under the microscope.

**Notes.**—<sup>1</sup>In the form of dilution of bouillon culture or suspension made in bouillon.

<sup>2</sup>The same procedure may be followed by using melted gelatin or agar, sowing, and allowing to set.

### **C4·18 REGENERATION<sup>1</sup> OF CULTURES.**

**Notes.**—<sup>1</sup>When old or moribund.

**C4·181.**—(1) Sow heavily<sup>1</sup> from the culture into bouillon as well as on agar or other solid medium. (2) Incubate.

**Notes.**—<sup>1</sup>To start growth.

**C4·182.**—(1) Add bouillon to the culture itself if growth has not been started by the usual methods of subculture. (2) Incubate.

**C4·183.**—(1) Sow heavily on pancreatin<sup>1</sup> agar (**M2·74**). (2) Incubate.

**Notes**—<sup>1</sup>The pancreatin or trypsin, especially if it be added to nutrient agar without any inactivation by sterilization, seems to favour growth.

### **C4·2 ANÆROBIC<sup>1</sup> CULTURE.**

**Notes.**—<sup>1</sup>Culture under conditions aiming at exclusion of the oxygen of the air. If this condition is satisfied, the methods described under aerobic culture are also applicable to anærobic culture.

### **C4·21 DISPLACEMENT OF AIR.**

**C4·211 BY HYDROGEN<sup>1</sup>.**—(1) Set up a hydrogen generating apparatus<sup>2</sup>. (2) Connect up the generator with wash bottles<sup>3</sup> containing in succession pot. permanganate<sup>4</sup> sol., alkaline pyrogallic acid<sup>5</sup>, and silver nitrate sol<sup>6</sup>. (3) Sow a culture tube<sup>7</sup> fitted with a rubber cork which carries 2 pieces of glass tubing<sup>8</sup>, the one the inlet tube passing down to the bottom of the tube, the other the outlet tube which does not project below the cork. (4) Connect up the inlet tube with the wash bottle system. (5) Seal off the outlet tube when the hydrogen is issuing pure<sup>9</sup>. (6) Apply a clip to the rubber tubing which connects the culture with the wash bottle system. (7) Cut

through the rubber tubing between clip and bottles. (8) Fill in the hollow portion of the rubber tubing down to the clip with melted paraffin. (9) Incubate.

**Notes.**—<sup>1</sup>Other gases which may be used in similar fashion are nitrogen, carbon dioxide and coal gas. <sup>2</sup>The hydrogen is generated by the action of 15 to 30 per cent. sulphuric acid upon granulated zinc. A Kipp's generating apparatus may be used or other similar apparatus. <sup>3</sup>All the wash bottle solutions are not absolutely necessary and the pot permanganate is often omitted. <sup>4</sup>To remove any organic matter. <sup>5</sup>To remove all traces of oxygen and neutralize any acid carried over from the generator. <sup>6</sup>To remove arsenic. <sup>7</sup>The methods with inlet and outlet tubes can be appropriately modified for culture of a number of tubes in a receptacle, or for plate culture. In such a case the receptacles, and not the tube, carries the inlet and outlet tubes. <sup>8</sup>Both pieces of tubing are bent at right angles close above the rubber cork and the outlet tube ends in a fine point which can be quickly sealed. <sup>9</sup>This should be tested for by collecting the hydrogen in a T.T. and igniting. If there is an explosion, there is still admixture of air with the hydrogen. If the sealing of the outlet tube is attempted before the hydrogen has displaced all the air, an explosion will take place.

**C4:212<sup>1</sup> BY EXHAUSTION.**—(1) Sow T.T. containing fluid or melted medium. (2) Heat the T.T. above the level of the fluid and draw the T.T. out in the blow pipe flame to form a thin neck. (3) Replace the wool plug with a rubber cork through which passes a glass tube. (4) Connect the glass tube to an exhaust pump with pressure tubing. (5) Lute the points of contact of the cork with T.T. and glass tubing with melted paraffin. (6) Exhaust of air. (7) Keep the T.T. in a water bath at 42°C to cause boiling of the medium under the reduced pressure<sup>2</sup>. (8) Seal the tube at the constriction. (9) Incubate. (10) Examine and identify growth.

**Notes.**—<sup>1</sup>The method is often combined with one in which the air is washed out of the culture tube by means of an indifferent gas such as hydrogen. <sup>2</sup>To drive out dissolved air from the medium.

**C4:213 BY EXHAUSTION.**—(1) Connect up a 2-way glass tap with a Y or T tube. (2) Connect up one remaining limb of the Y or T tube with a manometer system and the other limb with the culture apparatus. (3) Connect an exhaust pump by means of pressure tubing to one of the remaining limbs of the 2-way tap, and the laboratory coal gas<sup>1</sup> system to the other limb. (4) Set the exhaust pump going and open the gas tap. (5) Place the pump in communication with the culture receptacle by means of the 2-way tap. (6) Exhaust the culture apparatus of air. (7) Place the exhausted culture receptacle next in communication with the coal gas system by appropriately turning the 2-way tap. (8) Repeat the process of alternately exhausting the culture receptacle and filling it with coal gas as often as is necessary to

obtain thorough removal of air. (9) Exhaust the culture receptacle finally and leave it in a *vacuumized*<sup>1</sup> condition. (10) Incubate.

**Notes.**—<sup>1</sup>Or hydrogen generator. <sup>2</sup>The cultures will have been already sown. <sup>3</sup>Thus the bacteria are not grown in an atmosphere of coal gas. The coal gas is used to assist, by a process of washing out the air, in its complete removal. If the exhaust pump used is not very powerful, it is necessary to increase the number of vacuumizations which would otherwise be sufficient. The completeness and the maintenance of the vacuum may be controlled by using in the culture receptacle along with the sown cultures a T.T. of unsown medium to which has been added a few drops of 0.2 per cent. sod. sulphindigotate, 1 per cent. N-1 sod. hydroxide and 1 per cent. glucose. The blue colour given to the medium will be decolourized if the removal of air is sufficiently thorough. With a long incubation it will be necessary further to test the maintenance of the vacuum. To do so:—Connect up manometer and pump again with the culture receptacle. Empty all, the connections of air and note the position of the manometer needle. Then open the stopcock of the culture receptacle and judge from the movement of the manometer needle whether there is appreciable change in the maintenance of the vacuum.

**C4.214 BY HEAT.**—(1) Use freshly boiled and rapidly cooled fluid medium<sup>1</sup> in a T.T. (2) Sow. (3) Heat the T.T. strongly in the flame above the sown medium to expel the air. (4) Apply a sterile rubber cap quickly before the T.T. cools. (5) Apply melted paraffin to the cap<sup>2</sup> and upper part of the T.T. when cool. (6) Incubate.

**Notes.**—<sup>1</sup>The medium may contain deoxygenating substances such as glucose, 1-2,000 sod. formate, 1-200 sod. sulphite, etc. <sup>2</sup>The degree of vacuum obtained can be judged by the extent to which the top of the cap is sucked in.

**C4.215 BY BARRIER.**—(1) Fill a sterile T.T. one half its length with sterile glucose gelatin or glucose agar. (2) Boil the medium 3 min. to expel air. (3) Hold the tube in cold running water to cool its contents rapidly. (4) Place in a water bath at 42°C before the contents of the T.T. solidify. (5) Allow the T.T. and contents to attain this temperature. (6) Sow the melted medium with test material. (7) Hold the sown T.T. in cold running water to cause rapid solidification. (8) Incubate. (9) Break the T.T. at a suitable spot to obtain access to colonies. (10) Pick out the selected colony from the medium. (11) Carry out further examination<sup>1</sup>.

**Notes.**—<sup>1</sup>The microscopical appearance of the colonies may be made out by making transverse sections of the medium and examining the sections under a low power microscope.

**C4.216 BY BARRIER.**—(1) Add 0.25 gm. agar to 100 c.c. peptone bouillon<sup>1</sup>. (2) Heat 15 min. at 110°C to dissolve the agar. (3) Filter. (4) Distribute in T.T. to a height of about 5 cm. (5) Sterilize 15 min. at 120°C. (6) Sow with a pipette at the bottom of the medium

and along the track, or in several directions in the medium. (7) Incubate aerobically.

**Notes.**—The general character of the medium is that it is semisolid. Either gelatin or agar may be used. B. tetani, V. septique, etc., may be grown in this medium. All kinds of substances may be added to the medium to increase its cultural properties or for differential purposes such as glucose, lactose, maltose, mannite, serum, blood, bile, litmus, etc., provided that the consistence remains semisolid.

**C4:217 BY BARRIER.**—(1) Pour melted vaselin into a T.T. of bouillon or glucose bouillon to form a layer 3-4th in. deep. (2) Boil bouillon and vaselin 30 min. (3) Allow to cool. (4) Store for use. (5) Place in a water bath at 55°C at time of use. (6) Sow by means of a sterile pipette. (7) Incubate. (8) Examine and identify the growth.

**C4:218 BY BARRIER.**—(1) Sow by stab culture (C4:117). (2) Pour<sup>1</sup> a layer at least 8 mm. deep of oil, paraffin, vaselin, or simply fresh melted medium on top of the first medium.

**Notes.**—If the sowing has been done in a melted medium, this medium is allowed to set before the sealing layer is run on to the top of it.

#### C4:22 DEOXYGENATION.

**C4:221 BY PYROGALLIC ACID.**—(1) Place the cultures in a glass receptacle which can be rapidly made air tight<sup>1</sup>, along with the necessary<sup>2</sup> amt. of alkali and pyrogallie acid.

**Notes.**—<sup>1</sup>As for example a glass cover making contact by means of Ung. resinae. <sup>2</sup>1 grm. of pyrogallie acid and 10 c.c. 20 per cent. sod. hydroxide, or 10 c.c. 10 per cent pyrogallie acid and 2 grms. sod. hydroxide for each 100 c.c. of air space.

**C4:222 BY PYROGALLIC ACID.**—(1) Sow by stab (C4:117) freshly<sup>1</sup> sterilized glucose agar<sup>2</sup>. (2) Cover the upper surface of the agar with sterile melted vaselin. (3) Place the sown T.T. in a large receptacle<sup>3</sup> which is furnished with a rubber cork having both an inlet and outlet tube<sup>4</sup>. (4) Distribute pyrogallie acid in the bottom of the receptacle so as to present a large absorbing surface, and in sufficient quantity<sup>5</sup> for the air contained. (5) Close the receptacle with its rubber cork. (6) Exhaust the receptacle of air. (7) Clip the rubber connection with the exhaust pump. (8) Disconnect the apparatus from the pump. (9) Use the vacuum developed to draw in 20 per cent. sod. hydroxide on to the top of the pyrogallie acid. (10) Close the apparatus completely to the entrance of air. (11) Lute all the weak spots with melted paraffin. (12) Leave at R.T.<sup>7</sup> 2 to 3 hr. to allow of absorption of oxygen. (13) Incubate.

**Notes.**—<sup>1</sup>To ensure that the dissolved air removed by boiling is not reabsorbed before use. <sup>2</sup>Liquid media or surface sowing may be used. <sup>3</sup>The principle is

that used in a special bell jar apparatus (Bulloch) designed for the purpose. 'These tubes are capable of closure to the entrance of air, and one tube carries right to the bottom of the receptacle. It is by this tube the sod. hydroxide sol. is admitted. '1 gm. for 100 c.c. of air. '10 c.c. for each gm. of pyrogallie acid used. 'To allow oxygen to be absorbed before incubation in order to avoid growth of aerobic or facultatively anaerobic organisms.

**C4-223 BY PYROGALLIC ACID.**—(1) Use a strong glass T.T. 6 in. by 7-8th in. as enclosing T.T. and one of 5 in.  $\times$  7-16th in. as medium T.T. (2) Place 1 gm. dry pyrogallie acid powder in the bottom of the enclosing tube. (3) Boil the medium to be used in its T.T. (4) Cool rapidly. (5) Wrap 4 layers of thick blotting paper 4  $\times$  3 in., with pyrogallie acid between the layers, round the sown medium T.T. (6) Insert the medium T.T. with its layers of blotting paper into the enclosing T.T. (7) Pour 10 c.c. 20 per cent. sod. hydroxide sol. on to the pyrogallie acid in the outer tube. (8) Close quickly with a rubber cork. (9) Leave 2½ hr. before incubation to allow of deoxygenation. (10) Incubate.

**C4-224 BY PYROGALLIC ACID.**—(1) Cut a number of layers of blotting paper to the size of the internal area of a Petri dish. (2) Place the layers of blotting paper on a glass plate. (3) Surround the blotting paper with a thick ring of plasticine. (4) Soak the blotting paper in 10 per cent. pyrogallie acid<sup>1</sup>. (5) Pour on to the soaked blotting paper 20 per cent. sod. hydroxide. (6) Press down immediately a sown Petri plate on the plasticine ring. (7) Lute the whole well with added plasticine. (8) Allow time for absorption of oxygen. (9) Incubate.

**Notes.**—<sup>1</sup>Instead of soaked blotting paper crystals of pyrogallie acid may be used. Various special plates have been designed for this method of anaerobic culture.

**C4-225 BY PYROGALLIC ACID.**—(1) Sow a fluid culture in a T.T. (2) Push down the plug of wool, which should be non-absorbent. (3) Superimpose on the plug of wool a 2nd tight plug of absorbent wool. (4) Pour on to it 2 c.c. 20 per cent. pyrogallie acid followed by 2 c.c. 40 per cent. sod. hydroxide. (5) Close the T.T. with a well-fitting rubber cork or with a rubber cap<sup>1</sup>. (6) Incubate.

**Notes.**—<sup>1</sup>The absorption of oxygen will be shown by indentation of the rubber cap.

### **C4-23 DEOXYGENATION.**

**C4-231 BY GLUCOSE.** (1) Melt the agar in a deep 2 per cent. glucose agar T.T. (2) Add approximately 1-10th of its vol. of 1 per cent. sod. sulphindigotate<sup>1</sup>. (3) Keep the melted medium 5 min. at the temperature of boiling water. (4) Cool rapidly to 42C. (5) Sow. (6) Place the medium in the T.T. under running water

to produce rapid setting. (7) Cover the upper surface of the agar with sterile melted vaselin or with melted nutrient agar. (8) Incubate. (9) Wash the outside of the T.T. with 1-1,000 mercury bichloride followed by abs. alc. (10) Cut across<sup>2</sup> the T.T. at about the middle of the growth. (11) Detach the lower portion of the T.T. (12) Cut across the exposed medium at the desired point with a sterile knife. (13) Remove the growth with a platinum needle for examination and subculture.

**Notes.**—<sup>1</sup>Other reducing agents which may take the place of glucose and sod. sulph-indigotate are 0.5 per cent. sod. formate or 0.5 per cent. sod. sulphate. <sup>2</sup>On the colonies may be approached from above by pushing a platinum needle or capillary tube through the substance of the medium.

**C4\*232 BY HYDROGEN.**—(1) Fill the special<sup>1</sup> anaerobic jar with the culture tubes. (2) Heat the palladium asbestos capsule<sup>2</sup> in a Buusen burner. (3) Fix on the lid as quickly as possible. (4) Open the tap and slip on a piece of pressure tubing already attached to the hydrogen generator. (5) Turn on the gas and leave till there is no more oxygen left in the jar<sup>3</sup>. (6) Allow the apparatus to become cold. (7) Shut both taps. (8) Disconnect from the hydrogen generator. (9) Place the jar in the incubator.

**Notes.**—<sup>1</sup>A jar having a metal top with tap and tube leading in-out. The apparatus should be tested for tightness. This is best done by placing inside a few drops of ether and then fixing the lid. If the jar is then plunged under hot water, a leak will be detected immediately. <sup>2</sup>Asbestos wool covered with palladium black contained in copper gauze and attached to the lid. <sup>3</sup>The filling of the jar takes 15 min. during which time it requires no attention.

**C4\*233 BY HYDROGEN.**—(1) Fix a small piece of platinized asbestos at the end of a platinum wire. (2) Insert the other end of the wire into a short glass rod. (3) Insert the glass rod into a one-hole rubber cork. (4) Wrap up the whole arrangement and sterilize. (5) Remove water of condensation from an agar slope. (6) Sow. (7) Invert the tube and fill it by means of a sterile capillary pipette with hydrogen gas which has been passed in succession through 10 per cent. silver nitrate<sup>1</sup>, sulphuric acid<sup>2</sup>, pot. permanganate<sup>3</sup>, and 10 per cent. lead acetate<sup>4</sup> sol. (8) Heat the platinized asbestos for a moment in a free flame. (9) Insert the rubber cork with platinized asbestos firmly into the inverted tube. (10) Dip the end of the tube into melted paraffin. (11) Incubate.

**Notes.**—<sup>1</sup>To remove arsenic-impure hydrogen if the zinc used in the generator is not pure. <sup>2</sup>To remove water. <sup>3</sup>To oxidize organic matter. <sup>4</sup>To remove sulphur-impure hydrogen.

**C4·24 NIDUS<sup>1</sup> FORMATION.**

**Notes.**—<sup>1</sup>Involving the use of sterile fragments of tissue, such as brain, muscle, kidney, spleen, lymphatic gland, liver, boiled egg, etc. The tissue may be actually infected material and be used for sowing the medium, *e.g.*, gangrenous muscle. Fragments of vegetables, such as potato, also serve the purpose, as also cancellous substances, platinized carbon, spongy platinum, etc. Probably dependent to some extent on deoxygenation.

**C4·241.**—(1) Add with sterile precautions a sterile fragment of tissue to bouillon which has been freshly boiled. (2) Test sterility by incubating 48 hr. (3) Sow heavily<sup>1</sup>. (4) Place in the incubator without shaking the T.T.

**Notes.**—<sup>1</sup>A layer of sterile melted vaselin may be placed on the surface of the medium.

**C4·25 INDICATORS OF ANÆROBIOSIS.**

**C4·251.**—(1) Use for fluid media a T.T., along with the sown T.T., containing 2 per cent. glucose bouillon with N-500 sod. hydroxide and 1-100,000 methylene blue.<sup>1</sup>

**Notes.**—<sup>1</sup>If anaerobiosis is good and well maintained, the methylene blue is decolorized in 36 hr.

**C4·252.**—(1) Use for solid media a T.T. containing 2 per cent. nutrient agar with N-100 sod. hydroxide and 1-100,000 methylene blue.

**C4·3 PARTIAL ANÆROBIC<sup>1</sup> CULTURE.**

**Notes.**—<sup>1</sup>Equally well described as Partial Ærobie Culture. Syn. culture under reduced oxygen tension.

**C4·31.**—(1) Sow the culture T.T. (2) Place in a 10-litre jar in which carbonic dioxide is being generated by the interaction of 5 grm. sod. carbonate and 40 c.c. 1·8 sulphuric acid. (3) Allow the reaction to subside somewhat. (4) Seal the jar hermetically. (5) Incubate jar and contents.

**Notes.**—<sup>1</sup>About 10 per cent. of the air is replaced by carbon dioxide.

**C4·32.**—(1) Sow the culture T.T. (2) Connect up by means of rubber corks and rubber tubing to a culture T.T. freshly sown with *B. subtilis*<sup>1</sup>.

**Notes.**—<sup>1</sup>*B. megatherium* or *B. coli* may be used. The tubes of *B. subtilis* should be changed daily, if the incubation is long.

**C4·33.**—(1) Invert a sown Petri dish over another smaller Petri dish freshly sown with *B. subtilis* and resting on a glass plate. (2) Seal the inverted Petri dish to the glass plate hermetically with plasticine. (3) Incubate.

**C4·34.**—(1) Sow heavily a suitable medium. (2) Insert a sterile rubber stopper in place of the wool plug immediately after sowing. (3) Seal the T.T. hermetically. (4) Incubate.

#### **C4·4 EXAMINATION OF MATERIAL<sup>1</sup>.**

**Notes.**—The methods here given are of the nature of *examples* of culture methods. The full detail of the examination of particular material will be given under the heading of the material itself or under the heading of particular organisms and groups of organisms.

#### **C4·41.—BLOOD.**

**C4·411.**—(1) Let the patient lie on his back near the edge of the bed. (2) Cleanse the skin in front of the elbow joint thoroughly with soap and water. (3) Rub the skin with gauze or cotton wool soaked in ether, or alc., or acetone. (4) Cover with another piece of gauze or lint soaked in the same solution. (5) Allow the arm to hang out of the bed<sup>1</sup>. (6) Tie a bandage or piece of rubber tubing round the arm well above the elbow so as to cause the veins to stand out prominently. (7) Boil a 10 c.c. syringe in 0·5 per cent. sterile sod. citrate. (8) Take up into the syringe 0·5 c.c. 0·5 per cent. sterile sod. citrate. (9) Remove the gauze from the front of the elbow. (10) Pour a little alcohol or ether over the skin. (11) Make the puncture as the liquid is evaporating. (12) Pierce the vein in a direction nearly parallel to the surface of the skin. (13) Fill the syringe slowly with blood and withdraw it quickly. (14) Place the thumb on the puncture and raise the arm. (15) Release the bandage. (16) Sow 0·1, 0·2, 0·5, 0·75, 1 and 2 c.c.<sup>2</sup> of blood respectively in each of 6 bouillon T.T. (17) Shake to mix. (18) Incubate. (19) Examine the colourless clot above the sedimented erythrocytes for the development of colonies. (20) Enumerate colonies. (21) Isolate and identify colonies.

**Notes.**—<sup>1</sup>Rapid opening and closing of the fist will help to make the vein stand out. <sup>2</sup>Or sow 5 c.c. of the aspirated blood in 50 c.c. 0·5 per cent. glucose bouillon, and the other 5 c.c. in 10 c.c. sterilized ox bile. Both of these cultures are then incubated up to 7 d. and examined daily for fermentation, the production of gas and for the presence of motile organisms. If motile organisms are found, make subcultures on to neutral red bile salt agar and identify after incubation as organisms of the typhoid group. The paratyphoids ferment glucose and produce gas. B. typhosis produces no gas. Make subcultures from the bile in any case whether motile organisms are evident microscopically or not. If as much as 20 c.c. blood are added to 15 c.c. bile, the number of positive results is increased.

**C4·412<sup>1</sup>.**—(1) Sow 10 c.c. aspirated blood in large T.T. containing 40 c.c. each of 0·5 per cent. glucose bouillon. (2) Incubate up to 72 hr. if necessary. (3) Examine. (4) Make subcultures daily on to freshly

prepared blood agar or on to heated blood agar. (5) Incubate. (6) Examine and identify.

**Notes.**—<sup>1</sup>The method is applicable to the isolation of pneumococcus, streptococcus, *B. influenzae*, etc.

**C4·413.**—(1) Add 1 vol. sterile trypsin<sup>1</sup> sol. to 20 vol. bouillon. (2) Add 1 c.c. aspirated blood to 5 c.c. trypsinized bouillon. (3) Incubate. (4) Examine and identify any organism which develops.

**Notes.**—<sup>1</sup>The trypsin if not already sterile may be sterilized by filtration through a Berkefeld or Chamberland type of candle. Instead of adding the blood to trypsinized bouillon it may be added to simple trypsin sol.—4 vol. blood to 1 of trypsin sol. Subcultures are made from the mixture on arrival in the laboratory or it is first incubated as such.

**C4·414.**—(1) Add 10 c.c. aspirated blood to 10 c.c. ammon. oxalate sol.<sup>1</sup> (2) Make subcultures from the mixture on arrival in the laboratory.

**Notes.**—<sup>1</sup>Ammon. oxalate 1; sod. chloride 3; D. W. 500. Oxalate has less inhibitory effect on the growth of organisms than citrate.

**C4·415.**—(1) Add 1 vol. aspirated blood to 2 parts melted nutrient gelatin. (2) Allow the gelatin to set. (3) Despatch to a laboratory. (4) Place in incubator on arrival at 37°C. (5) Make subcultures on suitable media.

**C4·416.**—(1) Draw blood into a T.T. (2) Allow to clot. (3) Separate the clot from the glass. (4) Place in the incubator 2 hr. (5) Pipette off the serum for agglutination test. (6) Empty the broken up clot into a T.T. of sterile ox bile. (7) Incubate at least 48 hr. (8) Sow in appropriate media.

#### **C4·42 FÆCES.**

**C4·421 PROCURAL AND DESPATCH.**—(1) Send a small quantity<sup>1</sup> of loose<sup>2</sup> stool in a fæces tube<sup>3</sup> to the laboratory for examination as soon as possible<sup>4</sup> after the passage of the stool.

**Notes.**—<sup>1</sup>One cubic centimetre of a loose motion is ample for all bacteriological purposes. <sup>2</sup>The examination of a constipated motion in the case of a carrier is a worthless procedure, except in cases of chronic amœbic dysentery. <sup>3</sup>A small wide mouthed bottle provided with a cork. Inserted into this cork is a small spoon of white-metal having a bowl large enough to contain 1 gm. or a little more of faecal matter. <sup>4</sup>Dysentery bacilli are not, as a rule, recoverable after 6 to 8 hr. and other organisms likewise are not easily recoverable from a stool which has been passed for some time.

**C4·422 PROCURAL AND DESPATCH.**—(1) Make a suspension of the fæces as soon as possible with about double the vol. of 30 per cent. glycerinated 0·6 per cent. sod. chloride sol. (2) Send to laboratory for examination.

**C4:423 PROCURAL AND DESPATCH.**—(1) Add fine sterilized sand<sup>1</sup> to the faeces until a consistence of thick paste is obtained. (2) Despatch to laboratory.

**Notes.**—<sup>1</sup>Or finely powdered glass.

**C4:424 PROCURAL AND DESPATCH.**—(1) Pass a stout wire furnished with a sterile swab alongside the guiding finger into the rectum as high as possible. (2) Collect material from the wall of the rectum by means of a rotary movement in the same direction as the swab is rolled. (3) Withdraw with the same rotary movement. (4) Place the swab in a sterile T.T. containing a few drops of sterile water to prevent desiccation. (5) Send to laboratory for examination. (6) Make cultures at the latest within 4 hr.

#### **C4:43 FÆCES.**

**C4:431 TYPHOID GROUP ORGANISMS.**—(1) Make a suspension of 3 loopfuls of faeces in a T.T. of bouillon. (2) Leave 2 hr. at R.T. (3) Sow 1 loopful taken from the surface of the fluid on a 3½ in. plate of neutral red bile salt lactose agar (**M3:316**) or Endo medium (**M3:412** to **M3:414**). (4) Sow with a rectangular glass rod. (5) Incubate 24 hr. (6) Pick off colourless colonies. (7) Sow in a tube of sugar-free<sup>1</sup> bouillon in the morning. (8) Sow from the sugar free bouillon that same evening into glucose, lactose, dulcitol, saccharose and mannite sugar media. (9) Incubate. (10) Read the fermentation results on the following morning. (11) Sow, if the fermentations indicate it, on to agar slopes. (12) Read the fermentation results again after 48 hr. incubation. (13) Examine the organisms from the agar slope for motility. (14) Test the sugar-free bouillon cultures after 72 hr. incubation for indol. (15) Test suspensions in 0.85 S.S.S. from agar slopes for agglutinability with high titre sera.

**Notes.**—<sup>1</sup>Bouillon made with B-vit instead of fresh extract.

**C4:432 TYPHOID GROUP ORGANISMS.**—(1) Use T.T. of 10 c.c. 2 per cent. peptone water (**M4:11**) containing 1, 2, 35, 5 and 7 c.c. 1-10,000 brilliant green<sup>1</sup> with or without the addition of 0.4 c.c. 1-1,000 telluric acid in each T.T. (2) Make a suspension of 1 loopful faeces in 5 c.c. 0.85 S.S.S. (3) Transfer one large loopful of suspension to each T.T. of medium. (4) Incubate. (5) Examine and identify growth.

**Notes.**—<sup>1</sup>The typhoid-paratyphoid group are more resistant to the action of brilliant green than the common members of the coli group, except for a group of anaerobic fermenters. The latter however are very susceptible to the action of telluric acid. The medium is not suitable to the isolation of dysentery organisms.

**C4·433 TYPHOID GROUP ORGANISMS.**—(1) Shake up a piece of faeces about the size of a pea in 5 c.c. bouillon. (2) Keep 2 hr. at 37C. (3) Transfer 2 loopfuls of S. N. F. to a T.T. of peptone water containing 1-200,000 brilliant green and 1-25,000 telluric acid. (4) Incubate 18 hr. (5) Sow on neutral red bile salt lactose agar (**M3·316**).

**C4·434 DYSENTERY ORGANISMS.**—(1) Use freshly<sup>1</sup> passed faeces. (2) Pick up a loopful from a portion of faeces consisting of pus and mucus. (3) Wash well in 0·85 S.S.S. (4) Remove the material immediately with a platinum loop, allowing excess fluid to drain away. (5) Rub the material thoroughly on the surface of a bile salt lactose agar plate (**M3·316**). (6) Incubate 24 hr. (7) Pick off colourless colonies and sow in fermentation tubes of litmus lactose peptone water. (8) Incubate 12 hr. at 37C. (9) Examine and identify non-lactose fermenters.

**Notes.**—<sup>1</sup>If the specimen cannot be examined fresh, it should be placed in the ice chest.

**C4·435 V. CHOLERÆ.**—(1) Pick out 2 or 3 epithelial flakes. (2) Wash in several changes of 0·85 S.S.S. (3) Sow in T.T. of alkaline peptone water. (4) Incubate overnight<sup>1</sup>. (5) Pick up a portion of bacterial scum with a platinum loop. (6) Sow on the surface of alkaline blood agar plates (**M2·225**.) (7) Incubate 18 to 24 hr. (8) Examine and identify colonies.

**Notes.**—<sup>1</sup>Plates should be sown from the peptone water surface growth, if possible, at 6, 12 and 18 hr. after incubation. If the scum shows many contaminating bacteria, a fresh peptone water T.T. should be sown from the first before proceeding to plate sub-culture.

**C4·436 V. CHOLERA.**—(1) Pick out an epithelial flake. (2) Wash in several changes of 0·85 S.S.S. (3) Plant on to the surface of a poured alkaline blood agar plate (**M2·225**). (4) Incubate 18 to 24 hr. (5) Examine and identify colonies.

#### **C4·44 URINE.**

**C4·441.**—(1) Pass a sterile catheter. (2) Reject the first portion passed. (3) Receive the urine into a sterilized T.T. or flask. (4) Sow<sup>1</sup> a drop from the collected urine on agar and blood agar slopes and one drop on a litmus lactose agar plate.

**Notes.**—<sup>1</sup>The urine may be incubated 24 hr. before sowing, or again it may be added to bile medium and dispatched to a laboratory.

**C4·442 MALE.**—(1) Wash the glans penis and meatus thoroughly with hot soap and water everting the lips of the meatus to clean the

orifice. (2) Continue the washing with 70 per cent. alc. (3) Have the prepuce drawn back and the urine passed<sup>1</sup> in succession into 3 sterile receptacles. (4) Reject the first portion passed. (5) Sow from the urine of the 2nd or 3rd portion.

**Notes.**—<sup>1</sup>If the specimen can be obtained by sterile catheter so much the better. In the case of a typhoid infection it may be necessary to sow daily for a period of 10 d. to obtain a result. In gonorrhoea sow from pus or fish out floating filaments for sowing.

**C4-443 FEMALE<sup>1</sup>.**—(1) Have the genitalia thoroughly washed with hot soap and water followed by 70 per cent. alc. (2) Have the labia separated and the urine passed directly in succession into 3 sterile receptacles. (3) Sow from the 2nd or 3rd portion passed.

**Notes.**—<sup>1</sup>In the female it is very much better to have the specimen taken by means of a sterile catheter, although if this case is one of true bacteriuria, the infecting organisms are sufficiently numerous to exclude confusion from the presence of the contaminating organisms.

**C4-444 B. TUBERCULOSIS.**—(1) Mix purulent deposit with 4 per cent. antiformin. (2) Neutralize with 5 per cent. sod. sulphite and with 5 per cent. sulphuric acid. (3) Inject subcutaneously in a G.I.<sup>1</sup> (4) Knead the site of inoculation well. (5) Examine for enlargement of glands in 10 d. (6) Excise an enlarged gland and examine.

#### C4-45 SPUTUM.

**C4-451<sup>1</sup>.**—(1) Mix thoroughly equal vol. sputum and 30 per cent. antiformin in a T.T. (2) Remove a loopful of the mixture after 2, 3, 4 and 5 min., respectively, and spread over the surface of egg medium. (3) Furnish the T.T. with a rubber cap. (4) Incubate.

**Notes.**—<sup>1</sup>For isolation of *B. tuberculosis*. See also **C4-166** at 10 or 20 per cent. antiformin if the sputum is fluid.

**C4-452<sup>1</sup>.** (1) Have the sputum expectorated into a clean receptacle. (2) Examine it in a shallow porcelain dish or Petri plate. (3) Select a suitable portion. (4) Treat this portion with an equal vol. of 5 per cent. sod. hydroxide for 30 min. at 37°C. (6) Neutralize to litmus indicator with 5 per cent. hydrochloric acid. (7) Centrifuge. (8) Sow the deposit on egg medium. (9) Furnish the T.T. with a rubber cap. (10) Incubate.

**Notes.**—<sup>1</sup>For isolation of *B. tuberculosis*. Miscellaneous, *see* **C4-166** and **C4-451**.

#### C4-46 EXUDATE THROAT.

**C4-461.**—(1) Place the patient in a good light with fauces well illuminated. (2) Use a tongue depressor to get a good view. (3) Obtain material with a swab<sup>1</sup> from the surface of any visible membrane or from a part showing a suspicious appearance. (4) Return the

swab to its sterile T.T. and fix it in firmly. (5) Send it immediately to a laboratory. (6) Sow on serum medium (M2'35, M2'36). (7) Incubate for 12 to 18 hr. (8) Examine the colonies which have developed<sup>2</sup>.

**Notes.**—<sup>1</sup>Sterile absorbent cotton wool mounted on a piece of stout iron wire about 6 in. long. <sup>2</sup>*E.g.*, for *B. diphtheria*.

**C4'462.**—(1) Procure ox or horse serum<sup>1</sup> aseptically. (2) Add, with sterile precautions sterile 0·3 per cent. glucose sol. and litmus sol. to give a bluish tinge. (3) Distribute with sterile precautions in T.T. (4) Keep 3 hr. at 56°C on 3 successive days<sup>2</sup>. (5) Sow by plunging the swab with exudate material into the serum. (6) Incubate. (7) Examine after 6 to 12 hr. for the appearance of minute colonies. (8) Identify<sup>3</sup> the colonies.

**Notes.**—<sup>1</sup>Old antiteticanic or normal horse serum may be used but not anti-diphtheria serum. <sup>2</sup>The serum becomes syrupy in consistence. <sup>3</sup>The method is designed for the detection of *B. diphtheria*.

#### C4'47 BODY TISSUE.

**C4'471.**—(1) Perform a P.M. (2) Remove<sup>1</sup> the organs sterilely and place in sterile receptacles. (3) Sterilize the surface of the organ by searing it with a cautery or heated instrument. (4) Cut into the substance of the organ with a sterile knife through the cauterized area. (5) Sow from the substance or, as the case may be, contents<sup>2</sup> of the organ on suitable media. (6) Remove a portion of the organ pulp or its contents with a capillary pipette for further examination.

**Notes.**—<sup>1</sup>The operations of sowing and removal of material may also be done with the organs *in situ*. <sup>2</sup>Such as heart blood, intestinal contents, etc.

#### C4'48 WOUND SECRETION.

**C4'481.**—(1) Wash the wound well with sterile water or salt solution. (2) Pick up loopfuls of pus from the surface and from the depth of the wound. (3) Sow aerobically and anaerobically.<sup>1</sup>

**Notes.**—<sup>1</sup>The pus should not, as is sometimes done, be differentially heated before sowing anaerobically. Such treatment tends to bring out merely the more resistant type of spore bearing anaerobe, such as *B. sporogenes*.

#### C4'49 HAIR AND SKIN.

**C4'491 TINEÆ AND TRICHOPHYTA**<sup>1</sup>.—(1) Cut up or tease the test material<sup>2</sup> into as many pieces as possible with a sterile cutting needle. (2) Deposit small fragments of material upon the medium.<sup>3</sup> (3) Grow at R.T. (4) Subculture as soon as growth is visible.

**Notes.**—<sup>1</sup>See also C4'167. The trichophyta cannot be cultivated on acid media. Even if contaminating organisms are present the trichophyton growth is easily

differentiated, and especially by its tendency to grow down into the medium. *E.g.* hairs or squames. Use a glycerin medium of composition: Pepton 1; neutral pure glycerin 4; agar 1.5; D. W. 1,000. The agar may be omitted and the medium used in fluid form. The glycerin may be usefully replaced by maltose (**M4:522**, **M 4:523**) in the same proportion. Contaminating organisms have their growth inhibited by the large proportion of maltose in the medium, or if they do appear may be eliminated by the use of an exhausted medium (**C4:162**).

**C4:492 TINEÆ AND TRICHOPHYTA.**—(1) Place the test material in a moist chamber. (2) Keep at R.T. up to 7 d. (3) Watch for formation of mycelium. (4) Subculture as soon as growth is visible.

**Notes.**—<sup>1</sup>*E.g.*, hairs or squames.

#### **C4:5 EXAMINATION OF MATERIAL.**

##### **C4:51 MILK.**

**C4:511 ENUMERATION ORGANISMS.**—(1) Shake<sup>1</sup> the test milk well to break up clumps of organisms. (2) Make dilutions 1-100 1-10,000, 1-1,000,000. (3) Prepare plates of litmus lactose agar with 1 c.c. of each dilution. (4) Incubate 24 hr. (5) Enumerate<sup>2</sup> and identify the colonies which develop.

**Notes.**—<sup>1</sup>To mix. The cream contains more bacteria than the milk. <sup>2</sup>Total count, and relative proportion of lactose and non-lactose fermenters.

**C4:512 B. TUBERCULOSIS.**—(1) Centrifugalize 100 c.c. test milk at high speed. (2) Remove the cream layer. (3) Pipette off the milk layer until only about 4 c.c. remain in the centrifuge tube. (4) Shake up the residue of milk and the sediment. (5) Inoculate G.P. each with 2 c.c. subcutaneously, intramuscularly or intraperitoneally.

**Notes.**—<sup>1</sup>The use of larger quantities of milk and a considerable number of G.P. will increase the probabilities of detection.

**C4:513 B. DIPHTHERIÆ.** (1) Centrifugalize 50 c.c. test milk at high speed. (2) Remove the cream layer to a sterile T.T. (3) Pipette off the milk layer until only 1 to 2 c.c. remain. (4) Shake up the residue of milk and the sediment, and mix in also the cream. (5) Use this mixture for sowing suitable serum medium (**M2:35**, **M2:36**).

**C4:514 HÆMOLYTIC STREPTOCOCCI.**—(1) Add various dilutions of the test milk to 10 c.c. nutrient agar containing 1 c.c. rabbit blood. (2) Pour plates. (3) Incubate 48 hr. at 37°C. (4) Examine for hæmolysis. (5) Pick off those colonies showing a clear transparent, colourless zone and transfer to bouillon. (6) Sow in glucose, saccharose, raffinose, mannite, lactose, and salicin bouillon for determination of acidity, in milk for coagulation, and on blood agar plates for hæmolysis.

(7) Test virulence in G. P. by intraperitoneal injection of 1 c.c. of a 48-hr. bouillon culture and in mice by injection of 0.5 c.c.

**C4.515 CURDLING.**—(1) Incubate samples of milk in T.T. 12 hr. at 30 to 35C. (2) Examine.<sup>1</sup>

**Notes.**—<sup>1</sup>Good milk is sour and curdled, and there is a homogeneous coagulum present without much separation of curd or formation of gas. Coagulation in less than 12 hr. is abnormal and the absence of curdling in 24 to 48 hr. suggests the addition of preservatives or the presence of organisms inhibiting lactic acid formation, e.g., organisms of mammitis, putrefactive organisms, etc. If curdling is accompanied by an offensive odour, or if the coagulum is peptonized, the presence of *B. subtilis*, putrefactive bacteria, etc., is inferred. If all the samples show the same undesirable characters throughout, this points to a generalized condition of the stable. If only some samples show the appearance, this points to the cow which produces the sample of milk being at fault.

#### **C4.52 BUTTER AND CREAM.**

**C4.521.**—(1) Place a sample of the material in a T.T. (2) Keep at 42C till melted. (3) Sow to obtain discrete colonies from the melted material.

#### **C4.53 VEGETABLES.**

**C4.531.**—(1) Cut up the vegetable<sup>1</sup> finely with sterile scissors and forceps. (2) Add to a flask of sterile water<sup>2</sup>. (3) Shake vigorously. (4) Sow from the washings<sup>3</sup> in suitable media. (5) Incubate. (6) Examine the growths and identify.

**Notes.**—<sup>1</sup>If quantitative results are desired, a weighed quantity of vegetable should be used. <sup>2</sup>A definite volume, if quantitative results are required. <sup>3</sup>In definite amt., if quantitative results are required.

#### **C4.54 SOIL.**

**C4.541.**—(1) Make a suspension of 10 grm. soil, taken at from 2 to 6 in. below the surface, by grinding up finely and adding to 250 c.c. 0.85 S.S.S. (2) Shake well to obtain thorough suspension. (3) Dilute this suspension 1 in 250 with 0.85 S.S.S. (4) Sow 1 c.c. diluted suspension in 10 c.c. melted nutrient agar<sup>1</sup> (**M2.131**) and in 10 c.c. melted protein-free agar<sup>2</sup> (**M4.2**). (5) Pour plates. (6) Incubate 7d. at 18 and 22C.<sup>3</sup> (7) Make daily counts of the colonies developing. (8) Identify colonies.

**Notes.**—<sup>1</sup>Made with lemco. <sup>2</sup>Glucose 10; di-pot. phosphate 0.5; mag. sulphate 0.2; pot. nitrate 0.05; agar 20; water 100. <sup>3</sup>The lower temperature favours the appearance of the more slowly growing bacteria.

#### **C4.55 WATER.**

**C4.551 ENUMERATION BACTERIA.**—(1) Melt about 20 c.c. 2 per cent. nutrient agar. (2) Cool. (3) Place in a water bath at 42C and keep at this temperature till required for use. (4) Place 1 c.c. test

water or suitable dilution in a Petri plate. (5) Pour the melted agar into the Petri plate. (6) Rotate to mix. (7) Keep the lower portion of plate containing the agar inverted and resting on the cover for 1 hr. in the incubator to dry. (8) Incubate 24 hr. (9) Count the colonies which have developed.

**Notes.**—<sup>1</sup>V. also method with filter paper (C4-1132).

**C4-552 LACTOSE FERMENTERS.** (1) Add amt. of test water varying from 100 c.c. to .000001 c.c. to tubes of fluid bile salt lactose medium<sup>1</sup> (M3-316). (2) Incubate 48 hr. (3) Determine the smallest quantity of water which gives acid and gas in the incubated T.T.

**Notes.**—<sup>1</sup>Sol. taurocholate 0.5; peptone 2; lactose 1; litmus sol. to give purple colouration; D. W. 100. For amt. of water as large as 100 c.c. triple strength medium is used; 50 c.c. medium for 100 c.c. water. The 10 c.c. samples are added to 10 c.c. double strength medium. In America sol. taurocholate is not used in the lactose medium. It is also directed, instead of using triple strength, that "in every fermentation tube there must always be at least 3 times as much medium as the amt. of water to be tested. When necessary to examine larger amt. than 10 c.c. as many tubes as necessary shall be inoculated with 10 c.c. each."

**C4-553 PUTREFACTION TEST.**—(1) Convert 90 c.c. test water in a sterile glass stoppered bottle into a 1 per cent. peptone water by the addition of 10 c.c. 10 per cent. peptone. (2) Close with the stopper, (3) Incubate 48 hr. (4) Examine for the development of smell<sup>1</sup> of sulphuretted hydrogen.

**Notes.**—<sup>1</sup>Should be absent in a good water. Is present in a sewage polluted water.

## INDEX.

**AEROBIC**—CULTURE C41.

**AGAR**—SLOPE C4112.

**ALCOHOL**—CULTURE C4167.

**AMMONIA**—CULTURE C41661.

**AMMONIUM OXALATE**—BLOOD CULTURE C4414.

**ANÆROBIC**—CULTURE C41431, C41441, C42—ORGANISMS PRESERVATION C41431, C41441.

**ANÆROBIC CULTURE**—BARRIER C4216 to C4218—BUCHNER TUBE C42231—BULLOCH'S APPARATUS C42223—CARBON DIOXIDE C42111, C42121—COAL GAS C42111—DEEP TUBE C4215, C4216—DEOXYGENATION C422—DISPLACEMENT OF AIR C421—EXHAUSTION OF AIR C4212, C4214—GLUCOSE C42141, C4231—HYDROGEN C4211, C4232, C4233—LIBORIUS C4231—MACINTOSH AND FILDES C4232—NIDUS FORMATION C424—NITROGEN C42111, C42121—PALLADIUM ASBESTOS C4232—PARAFIN SEAL C4218—PARTIAL C43—PLATINIZED ASBESTOS C4233—PYROGALLIC ACID C4221 to C4225—SEMIFLUID MEDIUM C42161—TAROZZI C4241—TISSUE FRAGMENT C424—VACUUM C4212, C4213—VASELIN SEAL C4217, C4218—WASH SOLUTIONS C4211, C4233.

**ANÆROBIOSIS**—INDICATOR C42133, C425.

**ANIMAL**—INOCULATION C4169.

**B. ANTHRACIS**—ISOLATION C41642, C41643, C41691, C41692.

**ANTIFORMIN**—COMPOSITION C41661—CULTURE C41661, C4444, C4451.

**BACTERIA**—EROBIC CULTURE C41—ANÆROBIC CULTURE C42—CULTIVATION OF C4—ENUMERATION C41541, C41551—IN SPECIAL MATERIAL C44—ISOLATION V. ORGANISMS ISOLATION, AND CULTURE—ISOLATION SINGLE C4156 to C4159—PARTIAL ANÆROBIC CULTURE C43—PRESERVATION C44—REDUCED OXYGEN CULTURE C43—SUBCULTURE C414—VIABILITY C418.

**BARBER**—ISOLATION SINGLE ORGANISM C4159.

**BILE**—BLOOD CULTURE C44112, C4416—SALT LACTOSE MEDIUM C45521.

- BLOOD**—CULTURE IN FILE C4-112, C4-116—CULTURE METHODS C4-11—CULTURE TRYPSIN BOUILLON C4-113
- BLOOD SERUM**—AGAR PLATE C4-114—MEDIUM C4-162
- BLOWPIPE**—SEALING C4-131
- BODY TISSUE**—CULTURE FROM C4-17
- BRIGHT GREEN**—TYPHOID GROUP ORGANISMS C4-132, C4-133
- BUCHNER TUBE**—ANÆROBIC CULTURE C4-223L
- BULLOCH'S APPARATUS**—ANÆROBIC CULTURE C4-2223
- BURRI**—ISOLATION SINGLE ORGANISM C4-156
- BUTTER AND CREAM**—CULTURE C4-52
- CARBON DIOXIDE**—ANÆROBIC CULTURE C4-2111, C4-2121—PARTIAL—ANÆROBIC CULTURE C4-31
- CARRIER**—PECES EXAMINATION C4-4212
- V. CHOLERÆ**—ISOLATION C4-1612, C4-1614, C4-135, C4-136
- CELL**—ISOLATION SINGLE C4-156 to C4-159
- COAL GAS**—ANÆROBIC CULTURE C4-2111, C4-2121
- COLONY**—DISCRETE C4-1141, C4-1543—EXAMINATION C4-12
- CONDENSATION WATER**—AVOIDANCE C4-1133, C4-1151, C4-1162—REMOVAL C4-1132 C4-1221, C4-1558
- CULTURE**—ÆROBIC C4-1—ANÆROBIC C4-1431, C4-1441, C4-12, C4-2—ANIMAL C4-169—ANTIFORMIN C4-166—ALCOHOL C4-167—BLOOD C4-11—BUTTER AND CREAM C4-521—EXAMINATION C4-12—EXHAUSTED MEDIUM C4-162—EXUDATE THROAT C4-16—FACES C4-42, C4-43—FRACTIONAL C4-114 FROM HAIR AND SKIN C4-49—HANGING DROP C4-171—HEART BLOOD C4-172—KAOLIN C4-168—METHODS C4—MILK C4-51—OF MATERIAL C4-1—ORGANS C4-17—PARTIAL ANÆROBIC C4-3—PERMANENT C4-19—PLATE C4-113 to C4-116—PRESERVATION C4-114—TUS C4-18—REDUCED OXYGEN TENSION C4-3—REGENERATION C4-18—ROLL C4-119—SEALING C4-13—SELECTIVE FILTRATION C4-165—SELECTIVE HEAT C4-164—SELECTIVE INCUBATION C4-163—SELECTIVE MEDIA C4-161, C4-162—SERUM AGAR PLATE C4-114—SHAKE C4-118—SINGLE ORGANISM C4-156 to C4-159—SLANT C4-112—SLOPE C4-112—SOIL C4-54—SOWING C4-11—SPUTUM C4-45—STAB C4-117—STOCK C4-14—SUBSTANCE C4-115 to C4-118—SURFACE C4-112 to C4-114—UNSEALING C4-131, C4-132—URINE C4-44—VEGETABLES C4-53—VIABILITY C4-18—WATER C4-5—WOUND SECRETION C4-48
- DELEPINE**—DIETUM OF C4-1611
- DEOXYGENATION**—GLUCOSE C4-2141, C4-231—HYDROGEN C4-232, C4-233—SOD. FORMATE C4-2141—SOD. SULPHITE C4-2141—PYROGALLIC ACID C4-222 to C4-223
- DILUTION**—CULTIVATION C4-151 to C4-159—SUSPENSION C4-151 to C4-159—CELL C4-156 to C4-159

- B. DIPHThERiÆ**—ISOLATION C4461, C4462—MILK C4513.
- DRY**—AGAR SLOPES C41558—PLATES C41132, C4114, C4551.
- DYSENTERY GROUP ORGANISMS**—ISOLATION C44214, C4424, C4434.
- ENUMERATION**—ORGANISMS C41541, C41551—ORGANISMS MILK C4511—ORGANISMS, SOIL C4541—ORGANISMS, WATER C4551.
- EXHAUSTED**—MEDIUM C4162, C41673.
- EXUDATE THROAT**—CULTURE METHODS C446.
- FÆCES**—CARRIER C42212—V. CHOLERÆ C4435, C4436—CULTURE METHODS C442, C443—DYSENTERY ORGANISMS C44214, C44434—PRO-CURAL AND DESPATCH C4421, C4424—TUBE C44213—TYPHOID ORGANISMS C4431 to C4433.
- FILTRABLE VIRUS**—ISOLATION C41651.
- FORMALINIZED**—CULTURES C4149.
- FUNGI**—ISOLATION C41612, C41671.
- GAS GANGRENE**—ISOLATION ORGANISMS C41691.
- GONOCOCCUS**—PRESERVATION C41411, C41431, C41432, C41451—SUBCULTURE C41411—URINE C4421.
- GLUCOSE**—ANÆROBIC CULTURE C42141, C4231.
- GROWTH**—EXAMINATION C412—OBSERVATION OF C417.
- HÆMOPHILIC BACTERIA**—PRESERVATION C41411, C41431, C41432, C41451—SUBCULTURE C41411.
- HÆMOLYTIC**—STREPTOCOCCUS C4514.
- HAIR**—CULTURE METHODS C449.
- HANGING DROP**—CULTURE C4171.
- HEART BLOOD**—CULTURE C44712.
- HORT**—ISOLATION SINGLE ORGANISM C4158.
- HYDROGEN**—ANÆROBIC CULTURE C4211, C42121, C4232, C4233.
- HYDROGEN PEROXIDE**—CULTURE C41661.
- INCUBATION**—OPTIMUM C4163.
- INDIAN INK**—ISOLATION SINGLE ORGANISM C4156.
- INDICATOR**—ANÆROBIOSIS C42133, C425.
- B. INFLUENZÆ**—ISOLATION C41613, C44121—SUBCULTURE C41411—PRESERVATION C41411, C41431, C41432, C41451.
- INOCULATION**—ANIMAL C4169—CULTURE C411.
- ISOLATION**—ORGANISMS *V.* ORGANISMS ISOLATION.
- KAOLIN**—CULTURE C4168.
- LIBORIUS**—ANÆROBIC METHOD C4231.
- MACCONKEY**—DOUBLE STRENGTH MEDIUM C45521—MEDIUM M3316, C45521—TRIPLE STRENGTH MEDIUM C45521.
- MACINTOSH AND FILDES**—ANÆROBIC CULTURE C4232.
- B. MALLEI**—ISOLATION C41692.
- MATERIAL**—CULTURE C44.

**MEDIUM**—BILE SALT LACTOSE C4-5521—EXHAUSTED C4-162, C4-163—GLUCOSE LITMUS SERUM C4-162—GLYCERIN C4-163—PLATE C4-113 to C4-116—PRESERVATION C4-118—PROTEIN FREE C4-512—SELECTIVE C4-161, C4-162—SLOPE C4-112—SOWING C4-11.

**MENINGOCOCCUS**—PRESERVATION C4-141, C4-131, C4-132, C4-133—SUBCULTURE C4-141.

**MILK**—CULTURE C4-51—CURDLING TEST C4-515—B. DIPHThERIE C4-513—ENUMERATION ORGANISMS C4-511—HEMOLYTIC STREPTOCOCCI C4-514—B. TUBERCULOSIS C4-512.

**MONILIA**—ISOLATION C4-1812.

**NIDUS**—ANEROBIC CULTURE C4-29.

**NITROGEN**—ANEROBIC CULTURE C4-2111, C4-2121.

**ORGANISMS**—CULTIVATION OF C4—ENUMERATION C4-1541, C4-1551—ISOLATION F. ORGANISMS ISOLATION, AND CULTURE—PRESERVATION C4-14—SUBCULTURE C4-14—VIABILITY C4-18.

**ORGANISMS ISOLATION**—EROBIC C4-1—ANEROBIC C4-2—BLOOD C4-1—BUTTER AND CREAM C4-52—BY ALCOHOL C4-167—BY AMMONIA C4-1661—BY ANIMAL INOCULATION C4-169—BY ANTIFORMIN C4-166—BY DILUTION C4-151 to C4-159—BY FILTRATION C4-165—BY FRACTIONAL CULTIVATION C4-1614—BY HEAT C4-164—BY HYDROGEN PEROXIDE C4-1661—BY INCUBATION C4-163—BY KAOLIN C4-168—BY MEDIA C4-161, C4-162—BY PANCREATIN C4-1661—BY RAPID SUBCULTURE C4-1611—BY SOD. HYDROXIDE C4-1661—EXUDATE THROAT C4-46—FÆCES C4-42, C4-43—HAIR AND SKIN C4-49—HEART BLOOD C4-4712—MILK C4-51—ON EXHAUSTED MEDIUM C4-162—ORGANS C4-47—PUS C4-48—SINGLE C4-156 to C4-159—SOIL C4-54—SPUTUM C4-45—TINEA GROUP C4-491, C4-492—TRICHOPHYTON GROUP C4-491, C4-492—URINE C4-41—VEGETABLES C4-53—WATER C4-55—WOUND SECRETION C4-48.

**ORGANS**—CULTURE FROM C4-47.

**PALLADIUM ASBESTOS**—ANEROBIC CULTURE C4-232.

**PANCREATIN**—CULTURE C4-1661, C4-183.

**PARAFFIN**—SEALING C4-132.

**PARTIAL ANEROBIC**—CULTURE C4-3.

**PERMANENT**—CULTURE PREPARATIONS C4-149.

**PLATE**—CULTURE C4-113 to C4-116—CULTURE EXAMINATION C4-121—CULTURE PERMANENT C4-149—DRYING C4-1132, C4-114—SERUM AGAR C4-114.

**PLATINIZED ASBESTOS**—ANAEROBIC CULTURE C4-233.

- PNEUMOCOCCUS**—ISOLATION C4-1691, C4-1692, C4-4121—PRESERVATION C4-1411, C4-1431, C4-1432, C4-1451—SUBCULTURE C4-1411.
- POTATO**—BACILLUS C4-1643.
- PRESERVATION**—CULTURE C4-14—MEDIA C4-148.
- PURE**—CULTURE ORGANISMS C4-15, C4-16.
- PUS**—CULTURE C4-48.
- PYROGALLIC ACID**—ANÆROBIC CULTURE C4-222 to C4-225.
- REDUCED OXYGEN TENSION**—CULTURE C4-3.
- REDUCING AGENTS**—ANÆROBIC CULTURE C4-2141.
- REGENERATION**—CULTURE C4-18.
- RINGWORM**—ISOLATION ORGANISMS C4-1612, C4-1671.
- ROLL**—CULTURE C4-119—ISOLATION C4-153.
- RUBBER CAP**—SEALING C4-135, C4-1422.
- SEALING**—BLOW PIPE C4-131—PARAFFIN C4-132—RUBBER CAP C4-135' C4-1422—SEALING WAX C4-134—VASELIN C4-133.
- SEALING WAX**—SEALING C4-134.
- V. SEPTIQUE**—CULTIVATION C4-2161.
- SERUM**—AGAR PLATE C4-114.
- SHAKE**—CULTURE C4-118—CULTURE EXAMINATION C4-1221.
- SINGLE ORGANISM**—ISOLATION C4-156 to C4-159.
- SKIN**—CULTURE METHODS C4-49.
- SLANT**—CULTURE C4-112.
- SLOPE**—CULTURE C4-112—CULTURE EXAMINATION C4-122.
- SODIUM CITRATE**—BLOOD CULTURE C4-1441.
- SODIUM FORMATE**—ANÆROBIC CULTURE C4-2141.
- SODIUM HYDROXIDE**—CULTURE C4-1661, C4-452.
- SODIUM SULPHITE**—ANÆROBIC CULTURE C4-2141.
- SOIL**—CULTURE C4-541.
- SOWING**—CULTURE C4-11.
- SPORE BEARING ORGANISMS**—ANÆROBIC ISOLATION C4-164, C4-1691—ISOLATION C4-164.
- B. SPOROGENES**—CULTURE C4-4811.
- SPUTUM**—CULTURE METHODS C4-45—B. TUBERCULOSIS C4-166, C4-4511, C4-450.
- STAB**—CULTURE C4-117—CULTURE EXAMINATION C4-1221—DEEP C4-215.
- STREPTOCOCCUS**—HEMOLYTIC C4-514—ISOLATION BLOOD C4-4121—ISOLATION MILK C4-514—MEDIUM C4-514.
- SUBCULTURE**—STOCK CULTURES C4-14.
- B. SUBTILIS**—ISOLATION C4-1642, C4-1643.
- TAROZZI**—ANÆROBIC METHOD C4-241.
- TELLURIC ACID**—TYPHOID GROUP ORGANISMS C4-432, C4-433.

**TEST TUBE**—SEALING C413.

**B. TETANI**—CULTURE C42161.

**THROAT**—EXUDATE CULTURE C446.

**TINEA**—CULTURE C41612, C41671, C4491, C4492—MEDIUM C4493.

**TRICHOPHYTON**—CULTURE C41612, C41671, C4491, C4492—MEDIUM C4493.

**TRYPSIN**—CULTURE F. PANCREATIN CULTURE—BLOOD CULTURE MEDIUM C4413.

**B. TUBERCULOSIS**—CULTIVATION C41461, C41661 ISOLATION C41461, C41661, C41691, C41692, C4441—MILK C4512—SPUTUM C4451, C4452—URINE C4444.

**TYPHOID GROUP ORGANISMS**—BLOOD CULTURE C44112 BRILLIANT GREEN C4432, C4433—FECES C4431 to C4433—ISOLATION C41612, C41681, C4431 to C4433—TELLURIC ACID C4432, C4433.

**UNSEALING**—CULTURE C4131, C4132.

**VACUUM**—ANEROBIC CULTURE C4212, C4213—MAINTENANCE C4213.

**URINE**—CULTURE FEMALE C4443—CULTURE MALE C4442—CULTURE METHODS C444—B. TUBERCULOSIS C4444.

**VASELIN**—SEALING C4133.

**VEGETABLES**—CULTURE C453.

**VEIN**—PUNCTURE C4411.

**VIABILITY**—ORGANISMS C418.

**B. VULGATUS**—CULTURE C41642, C41643.

**WATER**—ENUMERATION ORGANISMS C4551—LACTOSE FERMENTERS C4552—PUTREFACTION TEST C4553.

**WATER OF CONDENSATION**—AVOIDANCE C4113, C41151, C41122—REMOVAL C41132, C41221, C41558.

**WOUND SECRETION**—CULTURE C448.

# VIRULENCE OF A MICRO-ORGANISM AND ITS DEPENDENCE ON THE CULTURE MEDIUM.

BY

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[Received for publication, May 25, 1921.]

THE problem of the virulence of a micro-organism is one which has not received very satisfactory treatment. The explanation of virulence as a capacity, under given circumstances, of rapid multiplication, or its explanation as some inherent property which is not dependent on rate of multiplication, are still matters of dispute. Then again the question of fixed virulence is obviously one which will find its elucidation in the explanation of the nature of virulence, or its reciprocal, susceptibility. Fixed virulence may be of two degrees that of high grade and that of low grade, and is a subject which is familiar to workers in Pasteur Institutes under the title of fixed rabies virus. In the case of rabies, fixation at a high level is effected by successive passages through a special animal, the rabbit. Workers at the subject of rabies also refer to the loss of virulence of rabies virus by successive passages through certain other animals. In this our contribution to the subject of virulence, the virus used by us was fowl cholera, and the investigation related to the maintenance or loss of virulence of the organism by weekly subculture on blood agar and upon ordinary tryptic agar made from mutton. The tryptic agar simply had rabbits blood added to it in the proportion of 10 per cent, while the tryptic agar had a reaction of pH 7.4 to 7.6. This note, it should be explained, relates to experiments on immunity as a result of inoculation, which cover the ground of effect of age and temperature on antigen, the use of avirulent and virulent strains for immunization, rate of loss and

rate of gain of virulence by cultivation on artificial media, the effect of immunization with allied organisms and the effect of immunization with wholly non-specific organisms. The strain to which this note refers was originally a virulent fowl cholera (*B. avisepticus*) which was grown by weekly subculture on blood agar and upon ordinary agar for 12 months before being subjected to final test. The growth on blood agar—and this is a point to be noted in connection with the explanation of virulence—is comparatively luxuriant, while that on ordinary agar was scanty.

The doses used are, throughout this paper, expressed as milligrammes of dried bacterial substance, and their estimation has been made from Brown and Kirwan's (1915) table through an opacity correlation. In this correlation we have taken an 11-fold dilution of a 1 per cent barium sulphate in citrated salt solution to be equivalent to 1 mgm. of dried bacterial substance.

The organism at its isolation was found to be extremely virulent to pigeons by intravenous inoculation. That this virulence could be 'fixed' by maintaining serial cultivation on blood agar is shown in the following table which represents trials of virulence on pigeons made at varying periods by intravenous injection throughout the 12 months.

TABLE I

*Showing the preservation (fixation) of virulence, with small variation around a point, throughout 12 months of weekly subculture on blood agar.*

Doses in milligrammes.						RESULT AFTER 72 HR. NUMBER OF PIGEONS.	
						Living.	Dead.
1	..	..	..	..	..	..	3
0.2	..	..	..	..	..	..	1
0.1	..	..	..	..	..	..	3
0.04	..	..	..	..	..	..	1
0.01	..	..	..	..	..	..	3
0.001	..	..	..	..	..	..	1
0.008	..	..	..	..	..	..	1
0.0016	..	..	..	..	..	..	1
0.001	..	..	..	..	..	..	3
0.00032	..	..	..	..	..	..	1
0.0001	..	..	..	..	..	..	2
0.00001	..	..	..	..	..	..	4
0.000001	..	..	..	..	..	..	16
0.0000005	..	..	..	..	..	..	12
0.0000001	..	..	..	..	2	..	35
0.00000005	..	..	..	..	4	..	16
0.00000001	..	..	..	..	7	..	32
0.000000005	..	..	..	..	3	..	13
0.000000001	..	..	..	..	9	..	15
0.0000000001	..	..	..	..	9	..	2

# 728 *Virulence of a Micro-Organism and its Dependence.*

The test of virulence of the strain of fowl cholera used is given in table II which shows how, when the organism is subcultured weekly on ordinary agar, it is at the end of 12 months, only able to kill in a dose of 1 mgm. whereas when subcultured on blood agar the virulence is maintained.

TABLE II

*Showing the comparison of the effect of growing one and the same strain of organism on ordinary agar and blood agar respectively.*

Dose in mgm.	GROWN ON ORDINARY AGAR.					GROWN ON BLOOD AGAR.				
	Weights of pigeons.	Result after, hr.				Weights of pigeons.	Result after, hr.			
		24	48	72	96		24	48	72	96
0.000000001 ..	340	..	..	..	alive	320	..	..	..	alive
	330	..	..	..	alive	480	..	..	..	alive
0.00000001 ..	390	..	..	..	alive	310	..	died	..	..
	330	..	..	..	alive	300	..	..	..	alive
0.0000001 ..	420	..	..	..	alive	270	..	died	..	..
	300	..	..	..	alive	290	died	..	..	..
0.000001 ..	330	..	..	..	alive	250	died	..	..	..
	310	..	..	..	alive	290	died	..	..	..
0.00001 ..	330	..	..	..	alive	320	died	..	..	..
	410	..	..	..	alive	300	died	..	..	..
0.0001 ..	320	..	..	..	alive	290	died	..	..	..
	350	..	..	..	alive	310	died	..	..	..
0.001 ..	340	..	..	..	alive	260	died	..	..	..
	280	..	..	..	alive	280	died	..	..	..
0.01 ..	350	..	..	..	alive	330	died	..	..	..
	320	..	..	..	alive	420	died	..	..	..
0.1 ..	270	..	..	..	alive	300	died	..	..	..
	320	..	..	..	alive	290	died	..	..	..
1.0 ..	200	..	died	..	..	300	died	..	..	..
	330	..	died	..	..	340	died	..	..	..

The "fixed" avirulence of strains grown for some time on ordinary agar instead of on blood agar was not fully taken up. The following table however indicates the probability of this being the case.

TABLE III

*Showing the fatal intravenous dose of two strains of fowl cholera which had been long subcultured on ordinary agar.*

STRAIN.	Pigeon.	Dose in mgm.	Result after 72 hrs.
M	1	1	dead
	2	.1	alive
	3	.01	alive
P	1	1	dead
	2	.1	alive
	3	.01	alive

## CONCLUSIONS.

1. *B. avisepticus* is an organism whose virulence can be maintained fixed within small limits without animal passage, by subculture on blood agar, while its virulence is very largely lost by subculture on ordinary agar.

2. There seems some probability that *B. avisepticus* when subcultured on ordinary agar becomes "fixed" in avirulence.

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# VIRULENCE OF THE ORGANISM AS A FACTOR IN THE EFFICACY OF PROPHYLACTIC VACCINES

BY

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[Received for publication, June 21st, 1921.]

IT is still matter of controversy whether the organism used in the preparation of a vaccine should be as virulent a strain as it is possible to obtain. The idea underlying the question is whether a vaccine prepared from a recently isolated or virulent type of organism may, quite apart from any considerations of amount to be administered, afford a higher protection than is attainable by the use of vaccine made from an organism which has been rendered avirulent by subculture.

It is evident, however, that this view is still in need of proof, for typhoid vaccines, as generally used, are made from a stock organism which has undergone subculture for many years.

Prophylactic plague vaccine on the other hand is prepared with special insistence on the need for use of a recently isolated virulent strain.

The experiments carried out by us are trials on pigeons of the efficacy of a fowl cholera antigen killed by heat, and injected intravenously at 7 days' interval. The doses used were two, the first of 0.5 mgm. of dried bacterial substance as determined from Brown's tables (1919) and

the second of 1 mgm. The use of fowl cholera was decided on because it had the advantage of being pathogenic to pigeons and so enabled us to use the more convincing protection experiments in our trials. The main trial is that of protection afforded by a vaccine prepared from the same strain of organism in its virulent and its avirulent state against the inoculation intravenously of a series of test doses of the living organism in its virulent condition. The organism when originally obtained was very virulent. This virulence was maintained, as explained in a previous paper (Harvey and Iyengar, 1921), by continued subculture on rabbit blood agar whereas the avirulent strain was obtained by continued subculture for 12 months on ordinary nutrient agar. The avirulent strain killed only in doses of 1 mgm. In addition to the evidence given by the protection trials we have that of the agglutination reaction obtained in a series of pigeons specially set apart for the purpose and not used in the protection test. The method of recording the agglutination is that advocated by Harvey (1920) and the two dilution values given as describing the limits of flocculation are practically those of complete agglutination and of macroscopically evident flocculation respectively. Those pigeons which had been treated with a vaccine prepared from the virulent and the avirulent organism had their serum each tested against both virulent and avirulent strains.

The control trials were carried out on 20 pigeons and occupied the day before as well as the day of the experiment, but are shown in a single table. This control was one designed to demonstrate the degree of virulence possessed by the organism used as test of protection, and also to give a basis for the conclusion as to the fact that protection had been afforded. Of the 40 pigeons set apart for the vaccine trials, fifteen in each category were used for the test of relative protection given by the vaccine prepared from the virulent and the avirulent organism and five each for the agglutination results from these two same strains.

In the control series 17 pigeons out of 20 succumbed to the test doses which were of a graded character. But it is to be noted that the dosage in this series ranged in both directions further than that in the series of pigeons receiving preliminary treatment with vaccine. If we take the range of dosage as being from 0.000,000,005 mgm. to 0.000,000,5 mgm. we find that all the pigeons out of the 14 used died in the control series as against 5 out of 15 in the series receiving virulent

organism vaccine and 6 out of 15 in the series receiving avirulent organism vaccine.

The following tables show exactly the data relating to dosage, weight of pigeons before and after dosage, survival and non-survival of test dose, and agglutination values of sera :—

TABLE I

*Showing the results of intravenous inoculation of a graded series of doses of living virulent B. avisepticus in pigeons.*

Serial No.	Weights in gm.	Dose in mgm.	Result.
1	300	0.000,000,000,1	lived.
2	320	"	"
3	290	0.000,000,001	dead in 24 hrs.
4	300	"	" " 48 "
5	320	0.000,000,005	" " 96 "
6	300	"	" " 24 "
7	300	0.000,000,01	" " 24 "
8	290	"	" " 24 "
9	300	"	" " 24 "
10	320	"	" " 24 "
11	290	0.000,000,05	" " 24 "
12	300	"	" " 24 "
13	300	0.000,000,1	" " 24 "
14	300	"	" " 24 "
15	290	"	" " 24 "
16	290	"	" " 24 "
17	320	0.000,000,5	" " 24 "
18	320	"	" " 24 "
19	290	0.000,001	" " 24 "
20	290	"	" " 24 "

TABLE II

*Showing the results of prophylactic intravenous inoculation of two doses (0.35 and 0.7 mgm.) of virulent and avirulent strains at intervals of one week, with test of protection by intravenous inoculation of a test dose of the living virulent strain 12 days after the last prophylactic dose.*

Strain.	Weights * in gm.			Test dose in mgm.	Result.
	1	2	3		
Virulent ..	310	310	310	0.000,000,005	lived.
	290	300	290	"	"
	320	310	320	"	"
	310	310	310	0.000,000,01	"
	300	290	300	"	"
	290	290	300	"	"
	310	300	310	0.000,000,05	died.
	290	290	290	"	lived.
	310	310	310	"	"
	320	320	310	0.000,000,1	dead in 24 hrs.
	290	290	300	"	" " 24 "
	300	300	300	"	lived.
	310	310	310	0.000,000,5	dead in 24 hrs.
	280	290	290	"	" " 24 "
	290	290	290	"	lived.
Avirulent ..	310	310	310	0.000,000,005	lived.
	320	310	320	"	"
	290	290	290	"	"
	300	300	300	0.000,000,01	"
	290	290	300	"	"
	300	310	310	"	"
	320	310	310	0.000,000,05	dead in 24 hrs.
	300	300	300	"	" " 24 "
	290	290	290	"	lived.
	310	300	310	0.000,000,1	dead in 24 hrs.
	310	310	290	"	" " 24 "
	300	300	300	"	lived.
	290	290	290	0.000,000,5	dead in 24 hrs.
	300	310	300	"	" " 24 "
	290	300	300	"	lived.

\* At the time of administration of the first dose, of the second dose and time of final test.

TABLE III

*Showing the results of test of agglutinating power of serum of pigeons inoculated intravenously with virulent and avirulent strains at intervals of one week with test 12 days after the last inoculation.*

Strain used in inoculation.	Weights.			AGGLUTINATING POWER.*	
	1	2	3	To virulent strain.	To avirulent strain.
Virulent ..	330	310	300	128 43-b	128 432-b
	300	300	290	128 432-b	128 432-b
	300	320	310	256 432-b	256 432-b
	290	280	290	128 432-b	128 432-b
	300	320	300	64 65-c 512 432-b	512 432-b
Avirulent ..	290	290	300	128 432-b	16 65-c 128 432-b
	300	300	290	32 65-c 512 432-b	64 65-c 512 432-b
	320	320	300	128 43-b	128 432-b
	300	290	290	128 432-b	128 432-b
	320	320	320	128 432-b	128 432-b

\* Where two values are given the upper is that of the limiting dilution of serum showing complete agglutination, the lower that of the limiting dilution showing macroscopically evident flocculation. Where only one value is given it is the second of these two.

## COMMENTARY.

The bacillus of fowl cholera is not one of the organisms which is used in the prophylactic inoculation of human beings. Deductions therefore made from the results here obtained must be based on the argument for analogy when transferred to the case of man and other organisms. The pigeons too used in trial are not immunized in the usual way by subcutaneous inoculation but by intravenous. They are in fact used simply as a means of measurement in life of response to prophylactic antigen and not specially as animals immunized with

suitable doses. It is to be noted however that the intravenous method of medication used in the trials is not only efficacious but has not brought about any disturbance of the subjects so far as it is to be gauged by the figures relating to weight.

#### CONCLUSIONS.

These are, in regard to the bacillus of fowl cholera, and with reference to the methods used in the trials :—

1. Protection afforded by prophylactic administration of an avirulent organism to infection by the organism in its virulent condition.

2. The protection afforded by the avirulent strain bears the proportion in these trials to that afforded by the virulent strain of 9 lives saved out of 15, as against 10 lives saved out of 15. The difference shown between the two is only slight and may or may not be really in favour of the use of the virulent strain.

3. The response to inoculation of an avirulent strain in the shape of agglutinating power of the serum is the same both for the avirulent and the virulent strain. The same result holds good for the inoculation of a virulent strain.

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# ON THE IMMUNIZING PROPERTIES OF ALLIED ORGANISMS AND NON- SPECIFIC ORGANISMS.

BY

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[Received for publication, July 16, 1921.]

IN a previous communication (Harvey and Iyengar, 1921), we showed that by intravenous immunization and test of pigeons the fowl cholera organism, *B. avisepticus*, showed no significant difference of effect whether it were virulent or almost completely avirulent. The present research conducted on exactly the same lines is a contribution to a solution of the questions of how far a group organism can immunize against another member of the group and how far an altogether non-specific organism can afford protection against infection. The organisms used were *B. avisepticus*, *B. cuniculisepticus* and *B. coli* of which the first was the specific organism, the second the group organism and the third the non-specific organism. The method adopted in all these studies is to give the prophylactic doses—in this case the equivalents of 0.5 and 1 mgm. of dried antigen intravenously and to follow the second of these inoculations 12 days later with a dose of the living test organism. The standardization of quantities used was effected by the method of Brown (1919).

The results of the trial were not quite what we expected. We had expected that the specific organism would give the highest protection, that the group organism would give the next highest degree of protection and that the non-specific organism would give the least or no protection. In these experiments however the results worked out to protection of the highest order from the specific organism and a more or less equal but considerably less protection from the group and non-specific organisms.

A control series of animals were inoculated with the living specific organism to show the mortality for the uninoculated.

TABLE I.

*Control Series. Showing the result of intravenous inoculation of pigeons with living B. avisepticus in varying doses.*

Serial No.	Weight in gram.	Dose in mgm.	Result.
1	280	0.000,000,005	lived
2	280	"	dead in 48 hrs.
3	300	0.000,000,01	" " 24 "
4	320	"	" " 24 "
5	300	"	" " 24 "
6	290	"	" " 48 "
7	290	0.000,000,05	" " 24 "
8	290	"	" " 24 "
9	300	0.000,000,1	" " 24 "
10	280	"	" " 24 "
11	280	"	" " 24 "
12	280	"	" " 24 "
13	280	0.000,000,5	" " 24 "
14	280	"	" " 24 "

TABLE II.

*Test Series. Showing the prophylactic effect of intravenous inoculation in pigeons of a specific organism B. avisepticus, a group organism B. carnisisepticus and a non-specific organism B. coli respectively against a series of test doses of the living specific organism also administered intravenously.*

Serial No.	*Weights.			Series of Pigeons.	Dose in mgm.	Result.
	1	2	3			
				SPECIFIC ORGANISM.		
1	300	280	280	"	0.000,000,005	lived
2	280	290	280	"	"	"
3	300	300	280	"	"	"
4	280	290	300	"	0.000,000,01	"
5	290	290	280	"	"	"
6	290	290	280	"	"	"
7	300	320	290	"	0.000,000,05	"
8	300	300	290	"	"	"
9	300	300	300	"	"	"
10	290	290	290	"	0.000,000,1	"
11	290	300	300	"	"	"
12	280	290	300	"	"	"
13	320	290	290	"	0.000,000,5	"
14	300	300	310	"	"	"
15	300	300	300	"	"	"

\*The weights (in grammes) given are those on the day of the first prophylactic dose, on the day of the second prophylactic dose and on the day of the test dose.

TABLE II.—*contd.*

Serial No.	*Weights.			Series of Pigeons.	Dose in mgm.	Result.
	1	2	3			
				GROUP ORGANISM.		
16	300	290	290	..	0.000,000,005	lived
17	290	280	290	..	"	"
18	300	280	290	..	"	"
19	320	300	290	..	0.000,000,01	"
20	300	300	290	..	"	"
21	300	300	300	..	"	"
22	300	290	300	..	0.000,000,05	dead in 24 hrs.
23	290	290	300	..	"	" " 48 "
24	300	290	300	..	"	" " lived "
25	320	300	290	..	0.000,000,1	dead in 24 hrs.
26	300	310	300	..	"	" " 24 "
27	300	300	290	..	"	" " 24 "
28	290	300	300	..	0.000,000,5	" " 24 "
29	280	300	300	..	"	" " 24 "
30	280	320	300	..	"	" " 24 "
				NON-SPECIFIC ORGANISM.		
31	300	290	300	..	0.000-000,005	lived
32	300	300	300	..	"	"
33	320	300	300	..	"	"
34	290	290	300	..	0.000,000,01	"
35	290	300	290	..	"	"
36	290	290	290	..	"	dead in 48 hrs.
37	300	300	320	..	0.000,000,05	" " 48 "
38	300	300	300	..	"	" " lived "
39	290	300	300	..	"	"
40	290	300	300	..	0.000,000,1	dead in 24 hrs.
41	290	290	300	..	"	" " 24 "
42	300	290	290	..	"	" " 24 "
43	300	290	300	..	0.000,000,5	" " 24 "
44	320	300	320	..	"	" " 24 "
45	300	300	290	..	"	" " 24 "

\* The weights (in grammes) given are those on the day of the first prophylactic dose, on the day of the second prophylactic dose and on the day of the test dose.

The two tables show for the same range of dosage :

(1) only one survival, and that for a very minute dose of *B. avisepticus* out of 14 uninoculated pigeons ;

(2) 15 survivals out of 15 pigeons prophylactically inoculated with *B. avisepticus* ;

(3) 7 survivals out of 15 pigeons for *B. cuniculisepticus* and *B. coli* respectively.

## CONCLUSION.

In this set of experiments with pigeons and with *B. avisepticus*

1. The prophylactic use of the specific organism showed a great saving of life (15 animals out of 15) as compared with a control series in which there was great mortality.

2. The prophylactic inoculation of an allied organism, *B. cuniculisepticus*, showed no evidence of any greater saving of life than did the prophylactic inoculation of a wholly non-specific organism.

3. Distinct evidence of protection by the prophylactic inoculation of a non-specific organism has been demonstrated.

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IYENGAR, K. R. K. . . . Virulence of the Organism as a factor in the efficacy of Prophylactic Vaccines., *Indian Journal of Medical Research*, 1921, Vol. IX, No. 4.

# IMMUNIZATION OR RESPONSE OF IMMUNIZED ANIMALS TO A SMALL DOSE OF ANTIGEN ADMINISTERED AT A LONG INTERVAL AFTER FIRST IMMUNIZATION.

BY

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[Received for publication, July 14, 1921.]

THERE is a great deal of literature now on the subject of anaphylaxia and sensitization. But although frequent statements are made regarding the close relationship existing between the immune state and the state of sensitization, comparatively little experimental work has been done to bring out exact quantitative relationships. If, for the sake of argument, we assume the sensitized individual to be an immunized individual then we must admit, having regard to our knowledge of the duration of sensitization, that an individual once immunized remains indefinitely sensitized. This fact is already taken advantage of in the case of various diseases by using tests diagnostically to produce obvious manifestations of the sensitized condition. Such tests are for the most part cutaneous, intracutaneous or subcutaneous. But it is legitimate to conclude that any method of introduction of appropriate antigen will serve to elicit the phenomenon of a response which differs quantitatively from the response obtainable in an unsensitized individual. The tests instead of being intracutaneous might, if that were convenient, be intravenous, intraperitoneal or intracerebral. Such tests are in fact used in animals in order to bring out marked manifestation of anaphylaxia. With minimal dosage the response in a sensitized animal is something less than that of anaphylactic shock. The dose may be reduced so as to give as slight a manifestation of sensitiveness as we please, a sensitiveness which nevertheless is quite distinctive.

In the domain of application we require :

- (1) Tests for the individual who has once suffered from a disease or has been immunized prophylactically to that disease.

- (2) Tests for carriers of an organism capable of producing disease in others.
- (3) Some idea of the dose of antigen and frequency of re-inoculation necessary in an individual who has once been the subject of prophylactic immunization.

Expressed in a different way these statements amount to this:—

- (1) Is it possible to detect by test, even if it be not possible to differentiate between them, individuals who have suffered from a disease, who have been inoculated against a disease, or who are carriers of the organism causing a disease? Can we say, as has been said for the persistence of the state of carriers—"Once immunized always immunized"? Direct serum tests are applied for the purpose, but have mostly been discarded, as they fail, or are supposed to fail, with the lapse of time. But is it possible that this failure may be due to insufficient appreciation of what constitutes a significant though slight difference from the normal? In other words have the serum tests used been carried out with sufficiently low dilutions of serum? Again it is possible that if an immunized individual is a sensitized individual, then the application of a minimal stimulus in the shape of appropriate antigen would produce a large and differential response.
- (2) Is it necessary for protection by re-inoculation of vaccine to use on the subsequent occasion as large doses of antigen as were used originally. If the response to the re-inoculation of a small amount of antigen is as great as the original response it would seem reasonable to suppose that the small amount was all that was required for the maintenance of immunity.

The experiments here given afford some data in answer to these questions. The trial consisted in the inoculation of 5 pigeons intravenously with the equivalent in suspension of 1 mgm. of dried bacterial typhoid antigen, and the examination of the serum of the pigeons first at 10 days intervals and then at 30 days interval throughout a period of 12 months for agglutinin titre. At the end of the 12 months a series of doses of antigen ranging from the original dose of 1 mgm. down to 0.01 mgm. were administered intravenously to the 5 pigeons, one dose to each, and their serum tested at intervals of 3 days for the agglutination

of agglutinins and for agglutinin titre. As controls to the results obtained there were used for similar tests 5 pigeons which had received no antigen at all and 5 pigeons which, not having previously received any antigen, received the same doses, 1 mgm. to '01 mgm. as the immunized pigeons. It is not possible to give all the tables presenting the degree of agglutination of serum for each dilution of serum from 1 in 4 up to 1-8192, although this was the series of dilutions tested throughout. The dilutions given are the *limiting* dilutions showing the maximum in which any agglutination at all was macroscopically evident, for the different intervals of trial. In addition the complete tables, with the mode of recording results which I adopt (Harvey, 1920), for uninoculated pigeons, the pigeons originally inoculated with 1 mgm. of antigen and then with varying doses after 12 months, and the pigeons inoculated only with the same varying doses, are given. The suspension used for agglutination test was one of living bacteria in suspension diluted so as to give the equivalent of 1 mgm. dried bacterial substance per c. c.

TABLE I

*Showing maximum dilutions (in the body of the table) in which macroscopically evident agglutination was exhibited by the serum of pigeons inoculated with 1 mgm. typhoid bacterial substance at intervals up to 12 months after the original inoculation.*

No. of days after inoculation	PIGEON.				
	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.
0	8	8	4	16	4
10	1,024	256	2,048	128	256
20	256	32	32	64	128
30	256	16	16	32	64
60	128	32	64	256	128
90	256	8	32	32	8
120	128	16	32	32	64
150	128	16	16	32	16
180	128	8	16	16	8
210	64	8	8	4	8
240	128	8	16	32	16
270	64	4	16	16	4
300	32	16	64	16	8
330	64	4	16	8	8
360	64	8	32	32	16

TABLE II

Showing agglutination results (in the body of the table) for a series of dilutions of serum in control pigeons receiving no antigen at all, in test pigeons 360 days after having received an intravenous dose of 1 mgm. of typhoid antigen, and in control pigeons 6 days after a varying dose of typhoid antigen.

Pigeons Description.	Dose in mgm.	No. of days after dosage to test.	DILUTION OF SERUM.									
			4	8	16	32	64	128	256	512	1,024	2,048
Control Pigeons uninoculated.	0	0	5432 b.	420 b.	o/a							
	0	0	320 b.	320 b.	o/a							
	0	0	20 b.	o/a								
	0	0	532 b.	532 b.	320 b.	o/a						
	0	0	320 b.	o/a								
Test Pigeons	1	360	654 c.	654 c.	654 b.	543 b.	5432 b.	o/a				
	1	360	543 b.	5432 b.	o/a							
	1	360	5 b.	43 b.	43 b.	320 b.	o/a					
	1	360	543 c.	543 c.	432 b.	30 a.	o/a					
	1	360	543 b.	432 b.	32 b.	o/a						
Control Pigeons inoculated	1	6	65 d.	65 d.	65 d.	65 d.	65 c.	65 c.	543 b.	320 b.	o/a	

TABLE II.—*contd.*

Pigeons Description.	Dose in mgm.	No. of days after dosage to test.	DILUTION OF SERUM.									
			4	8	16	32	64	128	256	512	1,024	2,048
Control pigeons inoculated ..	0.5	6	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{c.}$	$\frac{65}{c.}$	$\frac{5432}{b.}$	$\frac{50}{b.}$	o/a	
	0.1	6	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{654}{b.}$	$\frac{543}{b.}$	$\frac{432}{b.}$	o/a		
	0.05	6	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{5}{c.}$	$\frac{543}{b.}$	$\frac{5432}{b.}$	$\frac{40}{a.}$	o/a			
	0.01	6	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{d.}$	$\frac{65}{c.}$	$\frac{54}{b.}$	$\frac{5432}{b.}$	$\frac{540}{b.}$	$\frac{420}{a.}$	o/a	

TABLE III

*Showing maximum dilutions (in the body of the table) in which microscopically evident agglutination was exhibited 3, 6, 9, 12, 15 and 21 days after intravenous inoculation with varying doses of typhoid antigen in pigeons which had received a dose of 1 mgm. of the same typhoid antigen 360 days previously and in pigeons which had received no such previous inoculation.*

Pigeons.	Present dosage mgm.	Maximum aggluti- nation before present dosage.	No. of days after present dosage.					
			3	6	9	12	15	21
Having received 1 mgm. typhoid antigen 360 days previously ..	1	64	128	256	256	256	256	256
	0.5	8	256	2,048	256	64	32	16
	0.1	32	128	2,048	1,024	2,048	1,024	512
	0.05	32	512	8,192	1,024	2,048	128	256
	0.01	16	64	128	128	128	512	64
Having received no previous inocula- tion ..	1	—	64	512	128	256	128	64
	0.5	—	32	512	64	64	32	32
	0.1	—	16	256	512	256	256	256
	0.05	—	32	128	32	16	16	16
	0.01	—	8	512	64	32	16	1

## DISCUSSION.

The points which were to be elucidated by experiment were :

- (1) Whether an animal once immunized remained an animal always differentiated from one which had not been immunized ?
- (2) Whether an animal once immunized was more responsive to the introduction or application of antigen than one which had not been so immunized ?

With the help of some latitude in terminology the expression immunized animal here may be taken to cover the cases of the individual or animal which has once suffered from a disease, the individual or animal which has been once prophylactically inoculated, and the individual or animal which remains an active carrier after having once suffered from the disease. The number of animals used in the experiments here given are much too few to admit of any categorical answer to the questions. Moreover the only trial made was of an intravenous inoculation of the minimal doses designed to elicit a large response. Nevertheless it is reasonable to suppose that, with sufficient delicacy of the test applied, cutaneous, intracutaneous, subcutaneous, intraperitoneal, subdural, or other mode of introduction or application of antigen might be productive of similar effects. The practical applications of the thesis propounded here would be that direct evidence of the immunized state or, as the case may be, the sensitized state, should be found in all cases by direct investigation of serum reactions or by investigation of these reactions after introduction or application of a suitable and minimal dose of antigen. A little further extension of the idea contained in the thesis might lead us to suppose that quite a minimal dose of antigen would be sufficient to immunize an individual after he had once been immunized. If such be the case this would have far reaching effects on the practice of prophylactic vaccination. Instead of the repetition of the same large doses of prophylactic vaccine periodically all that would be required after the first immunizing inoculation would be repetition of quite minimal doses, with saving of quantities manufactured and saving of unpleasant reaction.

## CONCLUSIONS.

The conclusions given are to be differentiated from the 'discussion' given above as they are based strictly on the experimentation recorded.

1. The agglutination effect of intravenous inoculation of typhoid antigen in pigeons shows a diminution month by month after the inoculation. The drop is marked between the 10th and 20th day and very slow thereafter. The condition so far as a 12 months trial goes, seems to reach a constant low level which is however higher than that of the uninoculated animal.

2. The introduction intravenously of a dose of antigen 1-10th to 1-20th smaller than the original dose and even smaller still, calls forth an agglutinin response in the 12 months previously inoculated pigeons which is significantly greater than that elicited in uninoculated pigeons receiving the same doses.

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# RELAPSING FEVER IN THE PUNJAB.

( A PRELIMINARY REPORT.)

BY

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[Received for publication, July 25th, 1921.]

## CONTENTS.

	Page
I. INTRODUCTION .. .. .	748
II. PRELIMINARY CONSIDERATIONS .. .. .	749
III. THE EPIDEMIOLOGICAL CRITERIA OF RELAPSING FEVER .. .. .	754
IV. THE PRESENT EPIDEMIC, 1918-1920 .. .. .	757
V. THE HISTORY OF RELAPSING FEVER IN THE PUNJAB .. .. .	765
VI. GENERAL REVIEW .. .. .	773
VII. CONCLUSIONS .. .. .	776
APPENDIX A. The Provincial 'Fever' and Respiratory Indices of Relapsing Fever, 1867-1920 .. .. .	778
B. The 'Fever' and Respiratory Indices of each district of the Punjab during the years 1917, 1918, 1919 and 1920 .. .. .	780

## CHARTS.

CHART I .. .. .	The Provincial Monthly 'Fever' Mortality in the Punjab, 1920.
.. II .. .. .	The Provincial Monthly Respiratory Disease Mortality in the Punjab.
.. III .. .. .	The Provincial 'Fever' and Respiratory Indices, 1867-1920.

## MAPS.

MAP I .. .. .	The Distribution of Relapsing Fever in the Punjab during 1917.
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MAP	II	..	..	The Distribution of Relapsing Fever in the Punjab during 1918.
..	III	..	..	The Distribution of Relapsing Fever in the Punjab during 1919. ..
..	IV	..	..	The Distribution of Relapsing Fever in the Punjab during 1920.

## I. INTRODUCTION.

It is customary at the end of each year to prepare a summary of the main facts and figures bearing on the incidence of malaria in the Punjab during the preceding year. When the compilation of this account\* for the year 1920 was taken in hand it at once became apparent that something unprecedented during modern times had occurred in connexion with the mortality returned under the head of 'fevers.'

The abnormal nature of this mortality and the extent to which it indicates a departure from normal conditions will be rendered apparent by a scrutiny of Chart I. It will be seen that the monthly incidence of 'fever' mortality in 1920 is characterised by three abnormal features, viz:—

- (1) An almost complete absence of the usual autumnal rise in 'fever' deaths.
- (2) A phenomenal rise in the 'fever' deaths during May and June.
- (3) A relative rise, as compared with the three preceding months, in the 'fever' mortality in the month of December.

It is not proposed to consider here the first of these features since a study of the malarial circumstances prevailing during the year 1920 has conclusively shown that the absence of the normal rise in autumnal 'fever' mortality was due to the mild incidence of endemic malaria and the entire absence of epidemic malaria in all parts of the Province.

The remarkable rise in the 'fever' mortality during May and June, which, it will be shown, is a rare though not an unique feature of the 'fever' statistics of the Punjab, is so startling as to require explanation more especially from the point of view of the part played by malaria in its causation.

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\* NOTE :—The publication of these annual reports was discontinued by the Punjab Government in 1919.

The relative rise in the 'fever' mortality during the month of December, as compared with the three previous months, is also a rare occurrence whose significance is at present unknown.

In these circumstances a special study of the conditions prevailing during the year 1920 was at once undertaken as a matter of scientific interest and practical importance.

This report is concerned with the result of this investigation which, though preliminary in nature and restricted in scope, appears to have established the fact that a severe and widespread epidemic of relapsing fever occurred in the Punjab during 1920 which occasioned a mortality of not less than 26,000 and a morbidity of perhaps ten times this figure.

That an epidemic of this magnitude could have occurred without attracting attention—the presence of the disease was of course well-known—appears to render it expedient that all the facts so far ascertained should be placed on record without further delay.

## II. PRELIMINARY CONSIDERATIONS.

It is widely assumed that Indian vital statistics are of little or no value for scientific purposes on account of the nature of the agency employed in their compilation. It is contended with absolute truth—that no reliance can be placed on the accuracy of figures submitted by ignorant and often apathetic village watchmen—and it is held, with equal accuracy, that since the diagnosis of the cause of death is dependent upon the opinion of relatives or friends of the deceased or upon the whims of illiterate petty officials, the study of Indian diseases with the aid of mortality statistics is not only useless but it is actually calculated to give rise to erroneous conclusions.

Whilst admitting that these statistics contain many grave inaccuracies and that little or no reliance can be placed on the diagnostic powers of village *chamkardars*, it is necessary to combat the view that these defects, whose importance it is not desired to minimise, necessarily render the figures of no value in connexion with epidemiological investigations.

It is the more necessary to enter a protest against these facile criticisms of Indian vital statistics since there is reason to believe that they are responsible, more than any other circumstance, for the relative neglect, more especially in recent years, of epidemiological research in India.

Moreover, it necessarily follows, if these criticisms be well-founded, that since many generations must elapse before it will be possible to provide in each village or registration circle a competent agency for the collection of vital statistics, all efforts to elucidate the problems presented by Indian diseases, which involve the use of mortality figures, must be indefinitely postponed.

In these circumstances it is expedient to consider the subject in some detail with the object of showing that not only is this *non possumus* attitude towards Indian vital statistics not justified in the case of the study of epidemic diseases in the Punjab, but also of demonstrating that it is possible with the aid of admittedly imperfect figures to throw considerable light on the epidemiology of many diseases encountered in this Province.

There is reason to believe that although the total mortality figures recorded each year in the Punjab are not absolutely accurate, the error in defect more especially during the past twenty years, is not of sufficient magnitude to militate against their employment in epidemiological investigations. The Census Commissioner thus found in 1911 that the estimated population, as determined by adding the births and subtracting the deaths and allowing for the result of immigration and emigration during the previous decennium, corresponded closely with the actual population enumerated in that year. But the same degree of accuracy has not been attained throughout the past 54 years and it thus comes about that it is not possible to institute comparisons between the mortality rates during the early years of the registration of vital statistics and those now prevailing or to calculate the *mean* rates of mortality exhibited by different diseases over long periods.

But in spite of the varying and at one time great inaccuracy of the mortality figures it is still possible to trace the effect of epidemic diseases on mortality since the *fluctuations* in the latter will suffice to indicate their relative severity. It is thus concluded, in view of the great fluctuations in mortality occasioned by epidemic diseases, that existing inaccuracies in the mortality figures are not of sufficient importance to detract materially from their value for epidemiological purposes. It is even held that the older and less complete statistics are not altogether useless for this purpose.

But this view of the value of the mortality figures cannot unfortunately be adopted in the case of the classification of the cause of death which, as already stated, is of the crudest description. In the Punjab

the cause of death, as recorded in the death registers, is roughly classified for official purposes under one of the following heads:— 'Cholera,' 'small-pox,' 'plague,' 'fevers,' 'respiratory diseases,' 'dysentery and diarrhoea,' 'injuries' and 'all other causes.'

An examination of the mortality statistics over a long series of years, and more especially of the mortality caused by epidemic disease during the past 20 years, shows that, although cholera, small-pox and plague, which in typical form are well-known to and readily recognised by village watchmen, are in the main correctly recorded an epidemic of any of these diseases is almost invariably accompanied by a rise in mortality returned under other heads. Thus deaths from septicæmic plague are usually shown as being due to 'fever,' pneumonic plague is classified either under the head of 'fevers' or of 'respiratory diseases,' an epidemic of cholera affects the mortality recorded under the head of 'dysentery and diarrhoea' and, most marked of all, an epidemic of malaria is invariably accompanied by a great rise in mortality recorded under this last head ('dysentery and diarrhoea').

But by the use of appropriate methods it is possible, as will subsequently be shown in the case of relapsing fever, to allocate under their proper heads much of this mis-classified mortality and thus to determine, so far as epidemic diseases are concerned, the approximate mortality occasioned by them.

The main difficulty arises in connexion with the mortality from diseases classified under the head of 'fevers,' which, as is well-known, is an extremely comprehensive term, embracing all affections in which febrile symptoms form a conspicuous feature. Amongst epidemic diseases almost exclusively classified under this head in the Punjab are malaria, influenza, typhus, relapsing fever and enteric fever. In addition to these diseases the 'fever' mortality includes the deaths due to many other causes which do not readily lend themselves to classification under any other head. It is not at present possible and no attempt will be made to throw light on the true nature of the congeries of disease embraced in this portion of the 'fever' mortality, which, however, being relatively constant at all times, does not occasion any appreciable fluctuation in the monthly incidence of mortality.

But in the case of epidemic diseases (attended by mortality) their incidence, relative severity and seasonal periodicity will be exhibited by an abnormal rise in the monthly incidence of mortality. Thus in the Punjab, cholera is well known to be a hot weather disease which attains

its maximum usually in the months of July or August. Small-pox is usually most fatal in the months of May and June, bubonic plague in March and April, malaria in October and November and 'respiratory diseases' (mainly pneumonia) in December and January. Influenza is characterized by a spring and autumnal periodicity which is also the main season of mortality recorded under the head of 'dysentery and diarrhoea.' Relapsing fever, whose epidemiology will be more fully dealt with later, is a spring disease which in the Punjab is more especially associated with enhanced mortality in the months of May and June. Finally pneumonic plague and typhus are cold weather diseases, whilst enteric fever, which so far as is at present known is not a serious cause of mortality, is mainly encountered in the autumn.

The specific nature of any prevailing epidemic disease can usually be readily ascertained by field observations and laboratory investigation, and it thus comes about that, by the use of appropriate methods such as those which will shortly be detailed in the case of relapsing fever, it is usually possible to study its incidence and severity and to throw considerable light on its epidemiology in spite of difficulties occasioned by the unreliable nature of the vital statistics. It is, however, necessary to recognise that only the gross effects of mortality-producing epidemic diseases can be studied by methods designed on the above lines and it is also clear that these methods will be inapplicable in the event of two or more diseases prevailing in epidemic form concurrently.

It must also be emphasised that the precise nature of the epidemiological methods that it may be necessary to employ, as well as the interpretation to be placed on the facts thereby elicited, can only be properly undertaken in the light of an intimate knowledge—in the widest sense of the term—of local circumstances and conditions. It is, moreover, necessary to recollect that the mortality statistics should be treated fairly and that no attempt should be made to reach detailed conclusions—which their crude nature does not justify—in sole reliance on these figures.

Finally, it is necessary to mention one other point which requires to be taken into account in attempting to appraise, as the result of a consideration of vital statistics, the general state of the public health and relative incidence of individual diseases in the Punjab.

Owing to the fact that this Province is peculiarly liable to visitations of epidemic disease, which, by reason of their frequency, severity and variety, cause it to be equalled by few and probably surpassed by no

other country of equal size in the world, it is not possible by means of the methods ordinarily utilised for this purpose, to obtain comparable data with which to estimate the general state of the public health and the relative incidence of individual diseases.

Thus the great epidemic of influenza in 1918 so markedly affects the total death-rate and the 'fever' death-rate of any quinquennium in which the year 1918 is included as to render any comparison between the mean of this period and subsequent individual years fallacious if not actually misleading.

Similar remarks apply in the case of 'means' of periods comprising years in which other epidemic diseases prevailed, and it thus comes about that 'crude' *mean* figures, in so far as they are utilised for the purpose of affording information in regard to the state of the public health, are often *meaningless*.

Similar but even greater fallacies arise in connexion with the mortality caused by individual epidemic diseases, for, owing to defective methods of registration, it is usual for the latter to give rise, not only to the seasonal increase of mortality characteristic of the specific disease, but also to occasion an enhanced mortality attributed to other diseases. Thus an epidemic of malaria is invariably associated with a great relative increase in mortality recorded under the head of 'dysentery' and diarrhoea,' which circumstance not only causes the 'crude' mean of these diseases to be unduly enhanced, but—and this is of even more importance—it obscures the 'true' seasonal incidence of these diseases.

Similar difficulties arise in connexion with other epidemic diseases, one of which will be mentioned later in reference to the influence of relapsing fever in obscuring the 'true' seasonal incidence of 'respiratory diseases.'

It is not proposed to deal further with this subject here more especially as it is intended to prepare a detailed account of the methods by which it is thought many of the above difficulties in connexion with the interpretation of the vital statistics of the Punjab may, at any rate in part, be overcome.

But in spite of these limitations it is held that the vital statistics of the Punjab, if rightly considered and if properly utilised, are capable of throwing considerable light on the incidence of the many epidemic diseases which form so prominent a feature in the pathology of the Province.

It is thought that the epidemiological study of relapsing fever which forms the subject of this report aptly illustrates the nature of the methods by means of which useful and perhaps valuable information can be extracted from statistics which at first sight might appear to be so inaccurate as to render them wholly useless for any scientific purpose.

### III. THE EPIDEMIOLOGICAL CRITERIA OF RELAPSING FEVER.

Prior to 1920 relapsing fever is not known to have prevailed in epidemic form in the Punjab for many years and no great epidemic of this disease appears to have been described in India since 1877 when Vandyke Carter<sup>(1)</sup> carried out his classical observations on Spirillum Fever at Bombay.

From the clinical and epidemiological standpoint the disease is therefore less well known than formerly and it is thus not inexpedient to recall some of the characteristic features of the Indian form of relapsing fever as described by Vandyke Carter.

From the epidemiological standpoint relapsing fever differs markedly from most epidemic diseases by reason of its insidious onset, its slow progress and its gradual decline. It also exhibits a clearly defined seasonal periodicity. Thus in Bombay it was most prevalent during the months of March, April, May and June. Cragg,<sup>(2)</sup> as the result of some recent observations, concludes that epidemics of relapsing fever mainly occur in the United Provinces during the months of March, April and May. In the Punjab, it will be shown, both the accounts of old epidemics and the result of recent observations suggest a well-marked spring periodicity.

Other epidemiological points it is necessary to mention are the frequent association of famines with epidemics of relapsing fever and the peculiar liability of prisoners in jails to contract the disease.

Finally it is necessary to mention—although Vandyke Carter specially notes its absence at Bombay—there is a marked association between epidemics of typhus and relapsing fever although the former disease in the tropics and sub-tropics is essentially confined to localities exhibiting relatively cold climatic conditions. In such areas it is mainly a winter disease. Typhus fever therefore exhibits the same cyclical periodicity as relapsing fever but a different seasonal periodicity and a more restricted geographical distribution.

From the clinical aspect relapsing fever also presents some unusual features, since it is not attended by any of the dramatic symptoms

characteristic of cholera or plague nor indeed by any clinical signs which would cause its nature to be readily recognised. It is not proposed to detail the typical clinical picture presented by the disease, but it is necessary, for reasons which will shortly become apparent, to refer to a few points of diagnostic importance and epidemiological interest. It is in the first place desirable to emphasise the fact that the *relapsing* nature of the disease may never become apparent since Vandyke Carter found that death takes place in over 50 per cent of fatal cases about the 7th day either before or immediately after the first crisis.

Bronchitis is almost an invariable symptom and pneumonia, which Vandyke Carter states is very apt to be overlooked, occurs in about one-third of all fatal cases. It is of particular importance to note that pneumonia, when secondary to relapsing fever, exhibits the seasonal periodicity of relapsing fever (March—June) and not the seasonal periodicity of true lobar pneumonia. The writer has had occasion to note this fact in the Punjab, where outbreaks, ascribed to 'epidemic pneumonia' and 'pleuro-pneumonia' during the months of April and May, have been found by blood examinations to be due to relapsing fever.

Jaundice is by no means an invariable sign being present in only 21 per cent of all cases seen by Vandyke Carter during the years 1877—1879.

Finally, although relapsing fever is usually accompanied by well-marked constipation, a persistent diarrhoea is common in fatal cases towards their termination.

These clinical points suffice to suggest the liability of the disease, in the absence of blood examinations, to be mistaken for such febrile diseases as malaria, influenza, enteric fever, typhus and pneumonia. There is reason to believe that the disease has recently been diagnosed as one or other of these diseases. It has even been reported as yellow fever.

But the main reason for referring to this subject here is the fact that it exercises a direct bearing on the nature of the methods by which the true mortality occasioned by relapsing fever may be measured.

It is clear that if the true nature of the disease has been but rarely recognised by medical officers its occurrence amongst the general population would remain undetected. The vast majority of deaths from this disease would undoubtedly be recorded as due to 'fever,' a small proportion would be regarded as pneumonia and thus be classified

under the head of 'respiratory diseases,' whilst a few might be regarded as enteric fever and be recorded under the head of 'dysentery and diarrhoea.'

These facts afford material assistance in drawing up the 'epidemiological criteria' by means of which the incidence and severity of an epidemic of relapsing fever in the Punjab may be gauged.

In view of the seasonal prevalence of the disease in the spring and more especially of the enhanced mortality which it occasions in the months of May and June, it is permissible to assume that, in the absence of any other epidemic disease and in the known presence of relapsing fever (as determined by blood examinations), any abnormal rise in 'fever' mortality during the months of May and June is in the main the result of relapsing fever.

To obtain an index figure representative of the relative severity of the disease the percentage increase in the 'fever' mortality in May and June over that recorded in March and April has been calculated. It is found that in normal years this figure, which will be termed '*the fever index of relapsing fever*' varies between 5 and 10, whilst in years when relapsing fever is known to prevail in epidemic form it may be as high as 80 or more.

It has been decided to adopt this method of determining an index for various reasons although it is recognised that the disease also gives rise to mortality in March and April as well as in other months. In the first place other epidemic diseases, with the exception of small-pox, are rarely encountered in the months of May and June, the latter month more especially being a healthy month in the Punjab. It is true that the enhanced mortality due to such epidemic diseases as plague and influenza, which reach their maxima as spring epidemics in March and April, not infrequently affect the 'fever' mortality in May and possibly in June, but they are always relatively less prevalent in the two latter months. Thus an enhanced 'fever' mortality in May and June due to either of these epidemic diseases would occasion a low rather than a high 'fever' index of relapsing fever. Sources of error of this nature are therefore to a large extent eliminated. Other advantages attaching to this method of calculating an index of relapsing fever are the fact that it enables comparable indices to be calculated for the Province as a whole, for its component districts and even for registration circles. Finally it is possible to calculate the annual index figure for a long series of years in spite of changes in

population and variations in the degree of efficiency of the registration of mortality.

A similar index can be calculated on identical principles in the case of deaths classified under the head of 'respiratory diseases.' This index it is proposed to term the '*respiratory index of relapsing fever*.' It is indicative of and dependent upon the fact that an enhanced mortality from pneumonia during May and June is for the most part attributable to relapsing fever.

It is found that under normal conditions the mortality from respiratory diseases during March and April exceeds that recorded in May and June by about 10 per cent. In other words, in the absence of relapsing fever, the respiratory index is approximately -10. In the presence of an epidemic of relapsing fever the position is reversed, the deaths recorded under the head of 'respiratory diseases' in May and June exceed those reported in March and April and the respiratory index, instead of being a *minus* quantity, becomes a *plus* quantity.

An '*intestinal index of relapsing fever*' can be calculated on similar lines, but the figures for the diseases classified under the head of mortality due to "dysentery and diarrhoea" being relatively small, it has not been considered expedient to determine this index.

To sum up it is concluded that the epidemiological criteria of relapsing fever, the combined presence of which is essential to establish the occurrence of the disease in epidemic form in the Punjab, are as follows:

- (1) The absence of any other epidemic disease in the spring and the presence of a febrile disease which on investigation in selected localities can be shown on clinical and on microscopical grounds to be relapsing fever.
- (2) A 'fever' index of relapsing fever markedly in excess of normal.
- (3) A respiratory index of relapsing fever exhibiting a *plus* instead of a *minus* quantity.

#### IV. THE PRESENT EPIDEMIC, 1918-1920.

It is now proposed to examine, in the light of the epidemiological criteria of relapsing fever, the abnormal features presented by the 'fever' mortality during the year 1920.

The occurrence of sporadic outbreaks of relapsing fever in the Punjab during the years 1918, 1919 and 1920 is a well established fact.

The Punjab Sanitary Report for the year 1918 thus states that relapsing fever prevailed in a severe form in Gurgaon district in the extreme south-east of the Province and that the disease was apparently spreading in the neighbouring district of Karnal.

In March 1919 an outbreak of disease regarded as influenza was reported to have occurred in some villages in Karnal district, but the disease was subsequently proved on clinical and microscopical grounds to be relapsing fever. These outbreaks are stated to have caused 1,232 cases and 359 deaths (case mortality 29.1 per cent) during the year. Relapsing fever was also reported to have prevailed in the adjacent districts of Rohtak, Ambala and Simla during 1919. It is also stated that 29 cases of typhus fever occurred in Gurdaspur district and three cases of the same disease in Simla.

In the year 1920 relapsing fever was reported to have prevailed in numerous districts. Outbreaks occurred in Hissar district from May to July, in Ambala district during May, in Simla district from August to December, in Amritsar district during December and in Attock district from March to June.

From these facts, which are quoted from the Punjab Sanitary Report for 1920 (where a special prominence is accorded to the disease both from the epidemiological and administrative aspect) the *presence* of the disease and its unusual *prevalence*, more especially in the east and south-east of the Province, may be regarded as clearly and definitely established.

It is also clear that, so far as the plains are concerned, it exhibited the seasonal periodicity characteristic of the disease. It now remains to establish the absence of any other epidemic disease, more especially during the months of May and June, when the 'fever' mortality was exceptionally high. (*Vide* Chart I.)

That malaria was not responsible for the enhanced 'fever' mortality which occurred during the spring and early summer of 1920 is clearly proved as the result of a consideration of the epidemiological criteria of this disease. It will not be necessary to detail these here and it will suffice to mention two delicate tests by means of which an abnormal mortality due to malaria can at once be detected.

It has been found that an unusual prevalence of malaria is invariably followed by a rise in the spleen-rate of the affected area. If the enhanced mortality during the spring of 1920 had been occasioned by an epidemic of malaria the spleen rate in June 1920 should therefore show a

distinct increase as compared with the previous November. A scrutiny of the figures obtained as the result of the Provincial Spleen Census in November 1919 and June 1920, respectively, shows that the spleen-rate of school-children throughout all districts underwent a uniform decline during the above period and that the provincial spleen-rate in June 1920 was lower than it has ever previously been since the spleen census was first taken in 1913. The second test, the negative nature of which proves the absence of an unusual prevalence of malaria in the spring of 1920, is connected with the seasonal incidence and severity of infantile mortality. It has been found that the mortality of children under one year of age is immediately and greatly increased as the result of an epidemic of malaria. During 1920 the monthly incidence of infantile mortality was either normal or sub-normal throughout the year.

Plague was also extremely mild throughout the year 1920 and it was, in fact, entirely absent from many districts in which the 'fever' mortality in May and June was conspicuously high.

Influenza was present in a few areas in the plains of the Punjab during the winter and early spring of 1920, but there is little or no evidence that influenza was a cause of appreciable mortality during the hot period in May and June.

Certain outbreaks of disease regarded as influenza were reported to have occurred concurrently with an epidemic of relapsing fever in the districts of Karnal, Ambala and Hissar, but since in the case of the first-named district the epidemic was subsequently proved to be solely due to relapsing fever, it is probable other outbreaks attributed to influenza during May and June were in reality caused by relapsing fever.

It would therefore appear to be a justifiable conclusion that the 'fever' epidemic which prevailed in the Punjab during the spring of 1920, and more especially the abnormal mortality in May and June, was mainly if not entirely caused by relapsing fever. In these circumstances it becomes permissible to employ the epidemiological criteria of relapsing fever in order to throw light on the incidence and severity of the disease in the Province as a whole and in its component districts.

The provincial 'fever' index of relapsing fever was 4.2, a figure which, as may be seen by a reference to Appendix A, has only been exceeded four times during the past 54 years. The provincial respiratory

index is also extremely high, being +21·8. These figures therefore suggest that a severe and widespread epidemic of relapsing fever occurred in the Punjab during the spring of 1920.

Further light can be thrown on its geographical distribution and on its relative severity in different parts of the Province by means of calculating the 'fever' and respiratory indices of each district. The result of making these calculations is shown in Appendix B, but the facts contained therein can be more readily appreciated from a scrutiny of Table I in which the districts are arranged in accordance with the height of their 'fever' indices during 1920 and the three preceding years.

It will be seen that, assuming a 'fever' index of 20 or over connotes an epidemic of relapsing fever, 4 districts were involved in 1917 13 in 1918, 15 in 1919 and 18 in 1920.

The area involved in each of these years is shown in Maps I—IV, from which it will be seen that the epidemic appeared first in the extreme south-east of the Province (the districts of Gurgaon and Hissar) and in two central districts (Lyallpur and Shahpur) in 1917. In the following year, most of the eastern districts of the Province lying to the south of the Jhelum river became involved in the epidemic area. The year 1919 showed no marked change except that one district to the west of the Indus river was infected. In 1920, most of the southern and central districts were included within the epidemic area, in striking contrast with which was the continued freedom from infection of the districts in the north and north-west of the Province.

The mortality occasioned by the epidemic in 1920 may be roughly gauged by calculating the excess in 'fever' mortality in May and June over the mean mortality during these months.

The 'crude' mean 'fever' mortality for these two months during the period 1901—1917 is 60,509 which gives an excess in 'fever' mortality in the corresponding period in 1920 of 22,606.

But the 'crude' mean during the above period is much in excess of the 'true' mean owing to the inclusion amongst the 'fever' mortality figures of a large number of deaths from plague. If years in which great epidemics of plague be excluded it is found that, under present conditions of population, that the 'true' \* mean 'fever' mortality during May and June is approximately 50,000.

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\* The word 'true' is used in a strictly limited sense.

TABLE I.

*To show the 'fever' indices of relapsing fever in the districts of the Punjab during the years 1917, 1918, 1919 and 1920.*

Relapsing Fever index	1917	1918	1919	1920
Over 80	<i>Nil.</i>	Rodhak Kangra	<i>Nil.</i>	Lahore
Between 60-80	<i>Nil.</i>	Gurgaon Hoshiarpur	Rodhak Karnal	Rodhak Ambala Ludhiana Amritsar Gurdaspur
Between 40-60	<i>Nil.</i>	Karnal Gurdaspur Lyallpur Sialkot	Lahore Amritsar Lyallpur Sialkot Hissar	Karnal Kangra Lyallpur Sialkot Hissar Jullundur Sheikhpura Gujranwala Muzaffargarh
Between 20-40	Hissar Gurgaon Lyallpur Sialkot	Kangra Ambala Lahore Amritsar Hissar Ferozepur	Gurgaon Ambala Kangra Hoshiarpur Jullundur Gurdaspur Sialkot Dera Ghazi Khan	Hoshiarpur Ferozepur Montgomery
Under 20	23	14	12	10
TOTAL INFECTED	4	13	15	18

NOTE.—The district of Simla has been omitted as the figures are extremely small and the population is subject to great seasonal variation.

In 1920 a new district—Sheikhpura—was formed out of part of Lahore and Gujranwala districts.

In these circumstances it is concluded that the excess mortality due to relapsing fever in May and June 1920 (when plague was almost absent) was roughly 33,000. To this figure must be added the mortality from relapsing fever which is included in the mortality figures of respiratory diseases.

The 'crude' mean mortality for the months of May and June during the period 1905—1917 (the figures for the years previous to 1905 are not available) is 8,140, whilst the corresponding figure for the year 1920 is 11,595.

The excess in respiratory disease mortality which is ascribable to relapsing fever is therefore 3,455.

But the 'true' mean respiratory disease mortality during the above months, which is formed by omitting the years in which epidemics of plague or relapsing fever caused an enhanced mortality in May and June, is 7,426. The 'true' excess mortality in 1920 reported under the head of respiratory diseases in May and June is therefore 4,169.

It is thus seen that at the lowest computation the mortality ascribable to relapsing fever in May and June 1920 is 26,061, whilst if proper corrections be made it was approximately 37,000.

It is certain that even this latter figure under-estimates rather than exaggerates the true fatality of the disease during 1920, for no attempt has been made to include the deaths occurring in the remaining ten months of the year or to add the mortality reported under the head of 'dysentery and diarrhoea.'

Furthermore it is clear that the outbreak in 1920 only formed part of an epidemic which commenced in 1918. The deaths from relapsing fever in May and June of this year are calculated to have numbered approximately 19,000 and in the following year 7,000.

It is thus seen that the present epidemic of relapsing fever (1918-1920) has caused a mortality which a conservative estimate suggests was not less than 54,000, but which was probably in the vicinity of 60,000.

There is no means of determining the incidence of the disease amongst the civil population (the infection-rate), nor can the ratio of deaths to infections (the case-mortality) be given unless it be assumed that the case-mortality of 29 per cent ascribed to the epidemic in Karnal district in 1919 is representative of average conditions.

Vandyke Carter found that the case-mortality amongst Indians treated in hospital in Bombay during the epidemic in 1877-78 was 18 per cent, but many were debilitated as the result of famine. If it be permissible to assume that the case-mortality amongst untreated but not famine-stricken individuals in the Punjab during 1920 was 10 per cent, it would follow that something like 370,000 cases occurred in the Province during the above year.

That an epidemic of this magnitude should have occurred without its presence being widely recognised must be ascribed, partly to its insidious onset and almost imperceptible mode of spread, but mainly to the complete freedom of the epidemic from explosive characters and the absence of distinctive clinical features which permitted of the disease being regarded as one or other of the many affections embraced in the convenient but highly unscientific term 'fevers.'

It is now proposed to consider the abnormal features presented by the monthly incidence of 'fever' mortality which, as already stated, it was the original object of the present inquiry to investigate.

It can be confidently stated that the enhanced incidence of 'fever' mortality in the spring of 1920, and more especially the mortality in May and June, was due in the main to relapsing fever and to no other epidemic disease.

The 'fever' mortality in January was sub-normal as the result of the mild incidence of autumnal malaria in the previous autumn. A slight rise in mortality occurred in March and again in April which caused the 'fever' mortality in the latter month to be appreciably in excess of normal. In view of the great increase in mortality in May it is permissible to assume that the epidemic of relapsing fever which reached its maximum in the latter month commenced to exhibit an effect on mortality in the month of March.

It will be seen that whilst the mortality from the latter disease reached its maximum in May, the deaths in June were only slightly less.

In July the mortality declined rapidly, so much so that in the following month the 'fever' deaths were again approximately normal.

There is a slight rise, as compared with the previous month, in the 'fever' mortality in September, but the abnormally low mortality, as compared with the mean, in this and the two following months clearly indicates the absence of any fatal epidemic disease in these months.

In the month of December there is a distinct rise in the 'fever' mortality as compared with the two previous months, although the mortality is still greatly below the mean.

The nature of this enhanced mortality is not readily explainable, but it is interesting to note that Cragg,<sup>(2)</sup> quoting from an unpublished report by Brown, states that the latter was inclined to ascribe a similar rise in the United Provinces to relapsing fever.

An examination of the 'fever' mortality figures of the Punjab during the past 54 years shows that whilst the 'fever' deaths in December

are always greatly enhanced in years in which malaria is epidemic, they show a reduction as compared with the two preceding months. The December 'fever' mortality has, however, exceeded the deaths in the two preceding months on 15 occasions, of which all but one have been year-marked by exceptionally low autumnal 'fever' mortality. The exception was the year 1891 in which an epidemic of influenza was responsible for a high 'fever' death-rate during the last six months of the year.

On the other hand it is found, as a result of a scrutiny of the provincial 'fever' indices of relapsing fever given in Appendix A, that except in the years 1891 and 1920 (in both of which influenza prevailed in the winter), no relationship appears to exist between epidemics of relapsing fever and relatively high 'fever' mortality in December.

These facts therefore appear to suggest that whilst the mean December 'fever' mortality is largely composed of deaths due to malaria, it also includes a number of deaths from some other disease whose influence on the December 'fever' mortality only becomes apparent in years in which autumnal malaria occasions little or no mortality.

Some light is thrown on the nature of the diseases responsible for the enhanced 'fever' mortality in December 1920 by a scrutiny of Chart II in which the 'true' mean mortality from respiratory diseases is contrasted with the corresponding monthly figures for 1920.

The curves shown in this chart are of considerable interest. It will be seen that the mortality from respiratory diseases normally reaches its maximum in the months of December and January. During the next three months there are minor fluctuations which are followed by a small rise in mortality in the month of May.

In June there is a sharp decline, which is succeeded by consistently low mortality during the months of July, August and September. Finally during the last three months of the year the mortality from respiratory diseases steadily rises until it reaches its maximum in the month of December.

The corresponding mortality curve for 1920, in marked contrast to the above, exhibits an enhanced mortality from respiratory diseases in March and April followed by a conspicuous rise in May and June which closely resembles the rise in 'fever' deaths during the same period. (Chart I.)

It is justifiable to conclude that this enhanced mortality from respiratory diseases during May and June was for the most part due to deaths from relapsing fever with pneumonic complications.

It is thus seen that the observation of Vandyke Carter that pneumonia associated with relapsing fever exhibits the seasonal periodicity characteristic of the latter disease, rather than the seasonal periodicity of true pneumonia, is applicable to the Punjab.

Finally it is seen that the mortality from respiratory diseases was somewhat enhanced in December 1920 and that the curve of mortality during the last three months of the year exhibits a trend similar to that shown by the 'fever' mortality. (Chart I.) In these circumstances it seems probable that the rise in mortality exhibited by the 'fever' and respiratory disease curve in December 1920 was due to the unusual prevalence of a febrile disease exhibiting respiratory complications.

The nature of this disease or diseases cannot be stated, but in view of the facts already enumerated there does not at present appear any grounds for concluding that relapsing fever was responsible, whilst there is some reason to believe that influenza, which is known to have been prevalent during this month, may be partly concerned.

The liability of outbreaks of typhus fever to coincide with epidemics of relapsing fever, which has already been referred to and which will be shown shortly to be a conspicuous feature in the Punjab, renders it possible that an enhanced 'fever' mortality in December (in which month typhus fever mainly occurs) may be caused by the latter disease. In this manner it would be possible to explain an apparent high degree of correlation between enhanced spring mortality as the result of relapsing fever and high 'fever' mortality in December.

But beyond the fact that a few cases of typhus were reported during the year little is known regarding the incidence of typhus fever in the Punjab during 1920.

#### V. THE HISTORY OF RELAPSING FEVER IN THE PUNJAB.

Relapsing fever has long been believed to prevail in India, but it was not until 1852 that the identity of the disease with the relapsing fever of Europe was firmly established by Farquhar and Lyall as the result of an investigation at Peshawar.

As regards the Punjab nothing definite can be stated in regard to the period anterior to the year 1867 when the first formal annual report on the health conditions of the Province was published.

A perusal of the early Sanitary Reports, many of which contain detailed clinical descriptions of the disease, clearly shows that relapsing fever was regarded as being widely endemic throughout the Province and

that it occasionally assumed epidemic form both amongst the civil population and amongst prisoners in jail. Indeed medical officers of those days were well acquainted with and not a little puzzled by the frequent occurrence, sometimes in close conjunction, of two epidemic diseases, one of which was termed 'miasmatic fever' and the other 'contagious,' 'jail' or 'famine' fever. The first is now clearly recognisable as malaria, whilst there can be little doubt that the second was relapsing fever with which typhus fever was frequently intermingled and often confused.

It is only natural that the disease should have first come prominently to notice amongst prisoners in jails, since the latter were at that time the only section of the population of the Punjab whose state of health came under the constant supervision of European medical officers.

We thus read, in the Punjab Sanitary Report for 1868, of an outbreak of relapsing fever in the jail at Rawalpindi in 1862, in Multan jail in 1864, and a few years later in the jails at Ambala and Delhi. In 1868 an outbreak of a contagious febrile disease (which was not however contracted in the Punjab), occurred at Multan amongst a body of Punjabi muleteers returning from Abyssinia. Dr. Gray, the Civil Surgeon, who submitted a detailed report on the epidemic, definitely proved that the disease was relapsing fever with which he was well acquainted as it had 'prevailed epidemically in several of the Punjab jails in the years 1863, 1864, 1865 and 1866.'

From the year 1867 onwards numerous references to outbreaks of relapsing fever are made in the Annual Sanitary Reports of the Punjab, and it is now proposed to re-construct the history of the disease with the aid of the information contained in these reports which will be interpreted in the light of modern knowledge of the epidemiology of the disease and more especially in relation to the 'fever' and respiratory indices of relapsing fever.

In Chart III the provincial 'fever' indices for the years 1867-1920 are shown in graphic form together with the provincial respiratory indices for all the years for which the necessary data are available. The actual figures in both cases are given in Appendix A.

It will be clear that high 'fever' and respiratory indices do not necessarily connote the occurrence of an epidemic of relapsing fever, but it is thought that when, in association with high indices, the annual sanitary report contains an unequivocal reference to outbreaks of this disease, it is justifiable to assume that these figures, and more especially

the 'fever' indices, are indicative of the presence and relative severity of epidemics of relapsing fever. In order to emphasise this point the 'fever' index of every year in which an epidemic of relapsing fever is mentioned in the annual sanitary report is coloured red in Chart III.

The history of the disease during the past 54 years may thus be summarised:

1867. There is no mention of relapsing fever in the Sanitary Report, but the widespread occurrence of the disease, more especially in the Punjab jails, has already been mentioned. The 'fever' index is 24.7, which scarcely suggests the existence of any widespread epidemic. The data necessary to calculate the respiratory index are unfortunately not available.

1868. Except for an account of an outbreak of relapsing fever amongst Punjabi muleteers at Multan, which was not, however, contracted in the Punjab, there is no direct evidence in the annual report that relapsing fever prevailed in epidemic form amongst the civil population.

The 'fever' index and the respiratory index are both high, the former being 44.6 and the latter + 14.1, and are indicative of the occurrence of a widespread epidemic. In view of the gradual onset of epidemics of relapsing fever, and of the facts to be related in connexion with the following year, there is little room for doubt that these indices correctly reflect the incidence of relapsing fever.

1869. In this year a famine, which is well-known to be a frequent concomitant of an epidemic of relapsing fever, occurred in the Punjab. In May a severe outbreak of a febrile epidemic disease suddenly occurred and raged with special severity in the districts in the north of the Province. It declined somewhat in July, but again recrudesced in August, in some places before the onset of the monsoon.

In October and November, as the result of late and heavy rainfall in September, a severe epidemic, which was clearly due to malaria, prevailed in many parts of the Province and obscured and overshadowed the existence of any other febrile disease.

The Sanitary Commissioner at the end of the year caused special inquiries to be made in regard to the nature of the late epidemics, and, although Civil Surgeons almost unanimously attributed the unhealthiness of the year 1869 to 'intermittent' or 'miasmatic fever,' the Sanitary Commissioner was not able to accept the purely miasmatic theory of the origin of the late epidemic.

He points out that relapsing fever, as well as typhus fever, were well known to be endemic in the Punjab, and that these diseases had recently been shown to be unusually prevalent in the north of the Province.

He draws attention to the apparently contagious nature of the disease in some cases and its resemblance, mentioned by one Civil Surgeon, to the contagious fever which occurred amongst the Punjabi muleteers at Multan in the previous year. Stress is also laid on the fact that epidemics of pleuro-pneumonia, believed to be contagious, prevailed during the year in many of the Punjab jails and that the epidemic commenced *before* the onset of the monsoon.

These facts, and more especially the last, render it certain that malaria was not solely responsible for the unhealthiness of the year 1869 and it can be concluded with confidence that two distinct epidemics prevailed during this year, *viz.*, an epidemic of relapsing fever in the spring and an epidemic of malaria which commenced in the autumn.

The severity of the relapsing fever epidemic is indicated by a 'fever' index of 63·0—the highest on record—and a respiratory index of +8·8. The mortality caused by relapsing fever in the autumn cannot be stated as it was obscured by that caused by malaria.

1870. The year 1870 was even more unhealthy than the previous year. Throughout the winter and spring the 'fever' deaths remained abnormally high—contrary to the usual course of events after an epidemic of malaria.

This spring epidemic was particularly severe in the districts of Rawalpindi and Sialkot and led to special inquiries being instituted in these districts which led to the conclusion that the epidemic was either a severe form of intermittent fever or that it was relapsing or famine fever.

It is also worthy of note that outbreaks of contagious pleuro-pneumonia occurred extensively, more especially in the districts in the north and west of the Province, during the year.

These facts leave little room for doubt that the epidemic of relapsing fever which attained its maximum in the spring of 1869 recurred with considerable severity in the early months of the year 1870.

The 'fever' and respiratory indices were both approximately normal, but this circumstance is due to the fact that the enhanced spring mortality from both 'fevers' and respiratory diseases was almost equally

marked during the months of March, April, May and June. Whatever the explanation of this circumstance may be, it will be seen later that it is a feature invariably met with during the *decline* of an epidemic of relapsing fever.

1871. The Sanitary Commissioner states that relapsing fever still prevailed though with less severity than in the previous year. He states that 'the general sickness which broke out almost simultaneously all over the country in May 1869 continued to exercise an influence on the mortality returns until the end of the year 1871.'

The 'fever' index was nil, the mortality being equally high during March, April, May and June.

1872—1876. There is no mention of an epidemic of relapsing fever during these five years, the only reference to the disease being the remark that relapsing fever was present in Rawalpindi jail from September 1873 to March 1874. Typhus fever is also stated to have prevailed in this jail during 1872.

In 1872 the 'fever' index was slightly raised being 25·6, but the respiratory index was negative (—2·4) (2·4). From 1873—1876 the 'fever' index was either normal or subnormal.

1877. Famine conditions again prevailed widely in India during the year 1877. In this year the great epidemic of relapsing fever in South India, which formed the subject of Vandyke Carter's classical researches, took place.

There is no specific mention of an unusual prevalence of the disease in the Punjab, but it is noteworthy that after five years of silence the Sanitary Commissioner remarks that 'the relapsing or famine fever is familiar to the people of all parts of the Province and it often spreads over wide tracts in epidemic form commonly proving fatal by the development of jaundice, pneumonia or diarrhoea.' The 'fever' index is 22·1.

1878. An extensive epidemic of relapsing fever and typhus occurred in the Punjab during the year 1878. The Sanitary Commissioner states that the former disease proved especially fatal in the districts in the south-east of the Province, whilst typhus chiefly prevailed in the north and west of the Punjab.

The 'fever' index confirms the occurrence of a severe epidemic of relapsing fever, being 53·6.

1879—1881. The Sanitary Reports contain no reference to the disease during these three years but the spring 'fever' mortality

figures which in 1879 are greatly enhanced show a gradual decline to normal during the next two years. The 'fever' mortality during March, April, May and June was almost equally affected with the result that, as in 1870, the 'fever' indices are either extremely small or nil.

1882-1887. There is no mention of relapsing fever in the Sanitary Reports of any of these years.

The 'fever' indices are also normal or only slightly raised.

1887. Although relapsing fever is not referred to in the Sanitary Report, the 'fever' mortality figures are abnormally high both in the spring and the autumn. The autumnal 'fever' mortality was mainly caused by an epidemic of malaria. There is some reason to believe that the enhanced mortality in the spring was in part due to relapsing fever, since it was associated with an increase in the mortality returned under the head of 'chest diseases.' The Sanitary Commissioner remarks that the experience of 1887 suggests that there is some other factor or factors at work, not connected with rainfall, which is responsible for the enhanced mortality from 'fevers' in 1887.

The 'fever' index is, however, low (15·7), owing to the fact that the mortality was abnormally high in March and April as well as in May and June.

1888. There is no reference to relapsing fever in the Sanitary Report, but the 'fever' index is 30·8, the 'fever' mortality in June being particularly high, especially in the districts in the south-east of the Province. The Sanitary Commissioner states that a vast number of these deaths were probably due to pneumonia and bronchitis.

1889. The 'fever' index is 26·6. There is no reference to relapsing fever, but an unusual mortality from pneumonia was reported from many districts. Typhus fever prevailed in the districts of Peshawar and Rawalpindi.

1890. There is no reference to relapsing fever in the Sanitary Report, but the epidemiological circumstances of the year are obscured by the fact that in the spring of 1890 the Punjab was involved in the pandemic of influenza which prevailed almost throughout the world in this year. In view of the epidemic of relapsing fever in the following year there can be little doubt that part of the enhanced 'fever' mortality in May and June of 1890 ('fever' index 20·6) was due to the latter

disease. There was also an epidemic of malaria in the autumn. In these circumstances it is not a matter of surprise that the year 1890 was the most unhealthy in the Punjab since the registration of vital statistics was commenced in 1867.

1891. It is stated in the Sanitary Report that relapsing fever prevailed in the Punjab in epidemic form and that it proved more especially fatal during the early months of the year in the districts situated in the south-west of the Province. Typhus fever also prevailed as an epidemic, but this disease mainly affected the districts in the north-west of the Punjab.

Outbreaks of influenza also occurred mainly in the months of March and April, but in spite of this circumstance the 'fever' index of relapsing fever was 40·6.

1892. There is no reference to relapsing fever in the Sanitary Report, but the 'fever' mortality throughout the first six months of the year was even higher than in the previous year as the result of typhus in the winter and influenza during January, February and March.

The 'fever' index was consequently nil, but there can be little doubt that part of the enhanced 'fever' mortality in May and June was caused by relapsing fever.

1893-1899. Except for a reference to typhus fever in Peshawar and Kangra districts in 1894 no fact having any bearing on relapsing fever is given in the Sanitary Reports for these seven years.

The 'fever' indices are also normal or subnormal.

The 'fever' mortality in May and June in 1898 and to a less degree in 1899 shows a relative increase as compared with the three previous years.

1900-1902. Owing to a change in official procedure the Sanitary Reports from 1900 onwards become briefer and more formal documents which rarely deal with epidemiological considerations.

There is no mention of relapsing fever during these three years, but the 'fever' indices were 33·6, 34·9 and nil, respectively. Famine also prevailed in the Punjab during 1900 and there was some increase in mortality both from 'fevers' and pneumonia, more especially in the south of the Province, during the spring of 1902.

1903-1905. There is no mention of relapsing fever in the Sanitary Reports for these years and the 'fever' indices are also exceptionally low, a circumstance which is probably in part due to the widespread occurrence of epidemic plague in March and April.

From 1905 onwards the respiratory index of relapsing fever is also available, and since the figure for 1905 is—12·3 (the 'fever' index being nil), it is concluded that relapsing fever was not widely prevalent.

1906. There is only a brief reference to 'fevers' and no reference to relapsing fever in the Sanitary Report for 1906.

The 'fever' index (38·5) and the respiratory index (+8·5) both suggest that this disease prevailed extensively in the Province. An epidemic of plague, although of less severity than in the preceding or in the following year, occurred in the spring, but this disease disappeared by June and it does not therefore seem probable that the enhanced mortality from 'fevers' and respiratory diseases recorded in the latter month can be attributed to a misclassification of plague mortality.

There is therefore some reason to think that an epidemic of relapsing fever, which remained undetected largely owing to a concurrent epidemic of plague, prevailed in the Punjab during the year 1906.

1907. A great epidemic of plague was the dominant feature of the year 1907. The Sanitary Report contains only a scanty reference to 'fevers' and makes no mention of relapsing fever.

The 'fever' index was nil and the respiratory index was—7·5.

1908–1913. In spite of the occurrence of epidemics of malaria and plague the 'fever' and respiratory indices are consistently low and indicative of the absence of relapsing fever in epidemic form. The disease is not mentioned in any of the Sanitary Reports.

1914. The 'fever' index is somewhat high, being 27·7, whilst the respiratory index after being negative since the year 1906 again becomes positive (+10·4). The Sanitary Report states that an epidemic of relapsing fever occurred in Gurgaon district and that an outbreak of typhus fever was reported in Mianwali district.

1915–1917. There is no mention of relapsing fever in the Sanitary Reports for these three years, but the 'fever' and respiratory indices of the year 1915 both indicate the mild prevalence of the disease. The 'fever' index was 26·2 and the respiratory index was +4·6. In 1917 these indices were 3·3 and —28·0, respectively, but in spite of this circumstance the disease, it has already been shown, was mildly epidemic in four districts.

1918–1920. A detailed account of the history of the disease during these three years has already been given. It only remains to add that the information given in the Sanitary Reports in regard to the incidence

and relative severity of the disease is fully confirmed by the 'fever' and respiratory indices which are as follows :—

1918. 'Fever' index = 17.4. Respiratory index = + 6.8.

1919. 'Fever' index = 18.5. Respiratory index = + 25.0.

1920. 'Fever' index = 44.2. Respiratory index = + 21.8.

## VI. GENERAL REVIEW.

It is now proposed to review, in the light of the facts detailed in the two previous chapters, the main epidemiological features exhibited by relapsing fever in the Punjab during the past 54 years.

It is first necessary to appraise the value which may properly be attached to the 'fever' and respiratory indices as forming reliable data on which to base an opinion in regard to the occurrence of an epidemic of relapsing fever.

It will be seen from a scrutiny of Chart III that the occurrence of major epidemics of the disease, as indicated by the 'fever' indices, took place in the years 1869, 1878, 1891, 1906 and 1920. The respiratory indices are not available for the period 1872-1905, but in the years for which they are available (1869, 1906 and 1920) they afford confirmatory evidence of the occurrence of an epidemic of relapsing fever in these years.

The contemporary records contained in the Punjab Sanitary Reports it has already been shown, refer to widespread outbreaks of relapsing fever in the years 1869, 1878, 1891 and 1920, and it is equally important to note that they make no mention of epidemics in any other years.

It thus appears that in the year 1906 alone the Sanitary Report fails to confirm the 'fever' index, but in view of the concurrence of an epidemic of plague in this year, which exhibited almost the same seasonal periodicity as relapsing fever, it is by no means impossible that an epidemic of the latter disease would be overlooked.

In these circumstances it is concluded that the 'fever' index, more especially when confirmed by the respiratory index, may be regarded as possessing definite epidemiological value not only for the purpose of elucidating the cyclical recurrence of these epidemics, but also as a means of determining the distribution of relapsing fever epidemics and their relative severity in different parts of the Punjab.

It will be seen from the Chart that major epidemics of relapsing fever have exhibited a tendency to recur at more or less regular intervals

the number of years between each being 9 years, 12 years, 14 years and 13 years, respectively.

Furthermore, from the facts already detailed, it is clear that the attendant circumstances connected with these epidemics present many points in common.

Thus, although the *onset* of the epidemic may be sudden, its commencement must invariably be looked for two or three years previous to the year in which it attains its maximum. This circumstance occurred in connexion with the epidemics associated with the years 1869, 1878 and 1920, and it is probable that this feature was also present in the case of the epidemics in 1891 and 1906, but is obscured by reason of the fact that epidemics of influenza and plague occurred in the early spring of the years 1890 and 1905, respectively.

It has also been shown that epidemics of relapsing fever, in addition to being insidious in onset, are prolonged in duration so that they continue to exercise an influence on mortality (more especially in the spring) for two or more years after reaching their maximum.

The total 'wave-length' of these epidemics is thus about five years which comprises the epidemic year and the two years immediately preceding and following it.

It will be also seen from Chart III that, in addition to the main epidemic years, the 'fever' indices exhibit fluctuations in other years which in some cases are of sufficient magnitude to constitute minor epidemics.

It would scarcely be expected that with so elusive a disease as relapsing fever these minor epidemics would attract attention, but in the case of the year 1914, in which the 'fever' index and the respiratory index both suggest the occurrence of a minor epidemic, the Sanitary Report mentions the outbreak of the disease in one district. Similar confirmation of the occurrence of other minor epidemics is not forthcoming and it is therefore not possible to arrive at definite conclusions in these cases.

Nevertheless the available information suffices to show that relapsing fever has exhibited some degree of epidemicity in at least 27 years out of 54 years.

Unless therefore it be assumed that the disease is constantly being imported from other parts of India it must be concluded that relapsing fever is a disease which is permanently (at any rate since 1867) endemic in the Punjab and that the periodical epidemics are

CHART I.

The Provincial Monthly "Fever" Mortality in the Punjab.



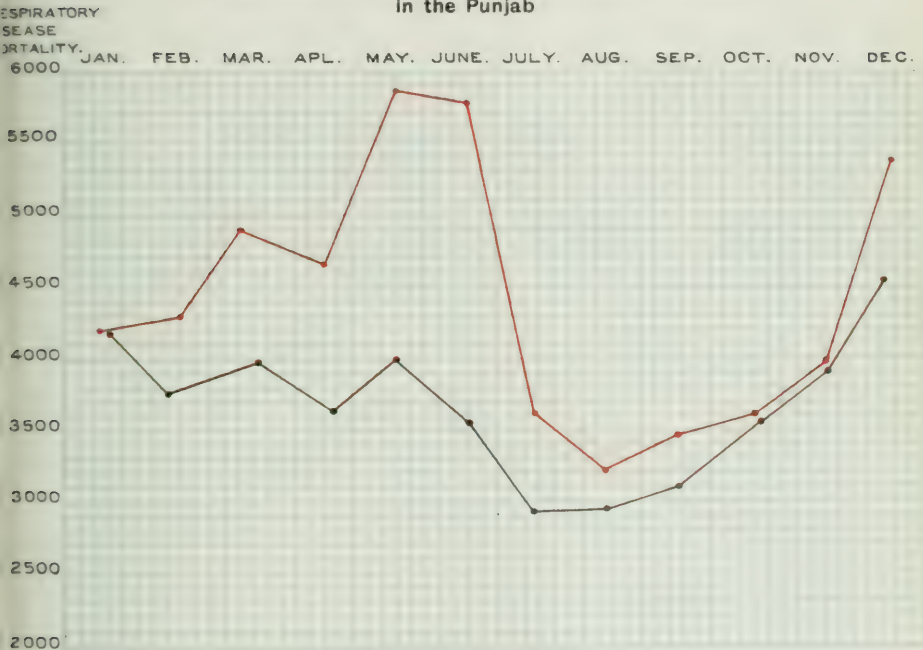
Mean monthly "fever" mortality 1901-1917 = black

Actual monthly "fever" mortality in 1920 = red



Chart II.

The Provincial Monthly "Respiratory Disease" Mortality  
in the Punjab

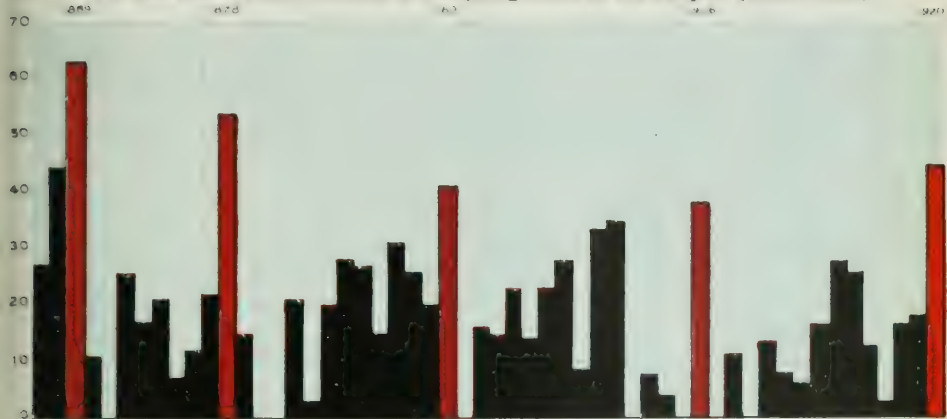


Mean monthly mortality from "respiratory" diseases = black  
Actual monthly mortality from "respiratory"  
disease in 1920 = red



Chart III.

To Show the "Fever" Indices of Relapsing Fever in the Punjab. (1887—1920)



To Show the "Respiratory" Indices of Relapsing Fever in the Punjab. (1887—1920)



(Columns coloured red indicate the years in which an epidemic of relapsing fever is recorded in the Punjab Sanitary Reports, except the year 1906.)



due to the increased toxicity and enhanced diffusibility of an endemic disease.

Another notable feature of these epidemics is their frequent though not invariable association with famine. Thus famine preceded and accompanied the epidemics in the years 1869 and 1878. The minor epidemic of 1900—if indeed the high 'fever' index in this and the following year is indicative of relapsing fever—was also associated with famine.

On the other hand scarcity did not prevail either in 1906 or in 1920.

The history of relapsing fever in the Punjab also serves to illustrate the well-known association of relapsing fever and typhus.

Thus during the quinquennium embracing the year 1869 numerous outbreaks of typhus were reported from the districts in the north and west of the Province.

This disease was also prevalent during the epidemic of relapsing fever in the year 1878, the same area as before being mainly involved. Identical circumstances occurred in connexion with the relapsing fever epidemic based on the year 1891.

There is no mention of typhus in connexion with the epidemics of 1900 and 1906, but typhus fever is again reported in association with the present epidemic of relapsing fever. (1918-1920.)

It will thus be seen that these two diseases exhibit an almost identical cyclical periodicity but differ somewhat in their geographical distribution, typhus fever being restricted to the montane and submontane districts in the north and west of the Punjab, whilst relapsing fever, although encountered in the same area, prevails extensively in the plains and appears to be more especially severe in the districts in the south-east of the Province.

The records also show that in another respect these two diseases present distinctive epidemiological features. Typhus fever appears essentially to exhibit a winter periodicity, being most frequently encountered in the months of December, January and February, whilst relapsing fever is mainly a spring disease.

The significance attaching to the peculiar relationship between these diseases will not be discussed here and it will suffice to remark that, whilst they possess the same cyclical periodicity, they exhibit distinctive characters in regard to their geographical distribution and seasonal periodicity.

The above account of the main epidemiological features presented by relapsing fever in the Punjab renders it possible to envisage the present epidemic in its true perspective and perhaps even in some small measure to forecast the course of events in the immediate future.

It is not possible to state whether the present epidemic reached its fastigium in 1920 or whether it will attain its maximum severity in 1921.\* In other words, it is not at present known whether the year 1920 corresponds epidemiologically to the year 1868 (which exhibited a similar 'fever' index) or to the year 1869.

It does, however, seem probable that the epidemic, which in 1920 had not appreciably affected the districts to the north of the Jhelum river, will in due course spread to these districts. And in the event of this contingency arising it is a justifiable deduction from the past history of the disease to anticipate that it will again be associated with an increased incidence of typhus fever.

#### VII. CONCLUSION.

It is not proposed to deal further with the epidemiological aspect of relapsing fever or to consider the precise rôle of the 'carrier' and the mode of spread of the disease, on which subjects the researches now being carried out by Major F. W. Cragg, M.D., I.M.S., may be anticipated to throw light.

The nature of the circumstances thought to be responsible for the cyclical recurrence and seasonal periodicity of relapsing fever will also be reserved for consideration in a separate paper now under preparation.

But it would stultify the ultimate object of all epidemiological investigations to ignore the practical conclusions arising out of the facts elucidated in this preliminary report.

It is clear that relapsing fever must now be regarded as a disease which is widely endemic in the Punjab and which at more or less regular intervals is apt to assume epidemic proportions. This fact, it is thought, requires more explicit recognition than it has hitherto received.

Owing to the absence of dramatic clinical features in relapsing fever and, it is believed, the not infrequent occurrence of mild and atypical cases, the diagnosis of the disease is apt to present great difficulties.

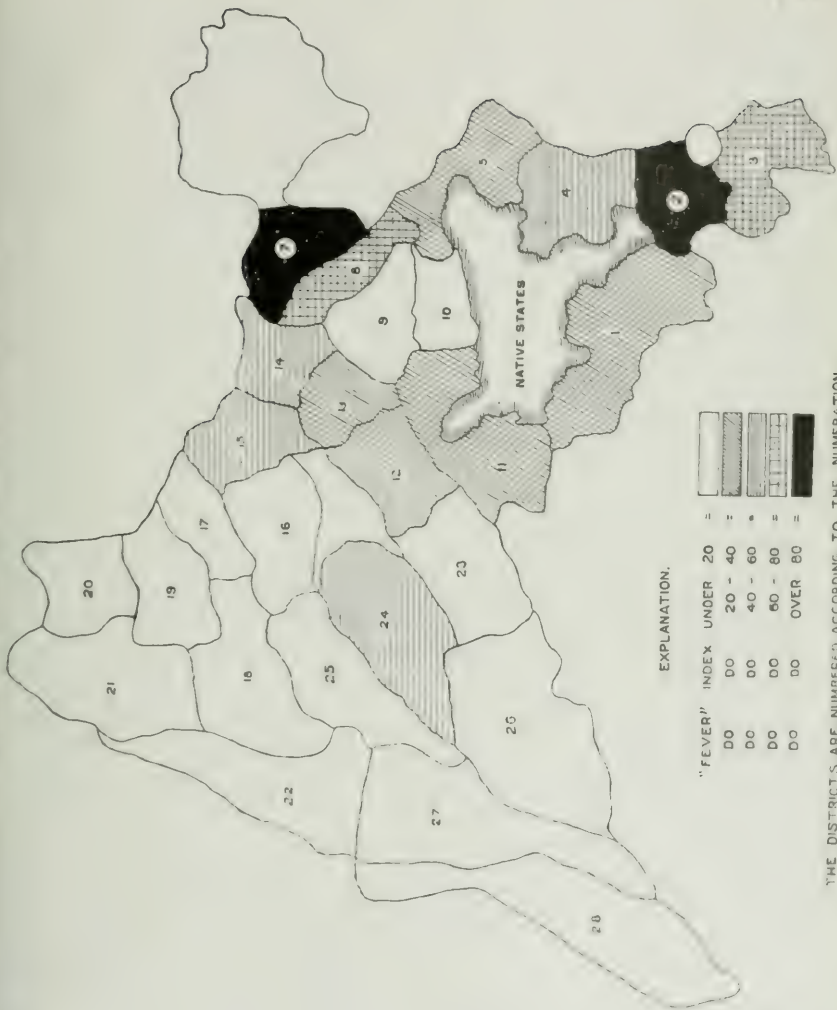
It is therefore necessary that the possibility of relapsing fever should be borne in mind in all obscure cases of 'fever' and that steps should be

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\* NOTE—It has since been determined that relapsing fever was less widespread and severe in 1921, than in the former year.





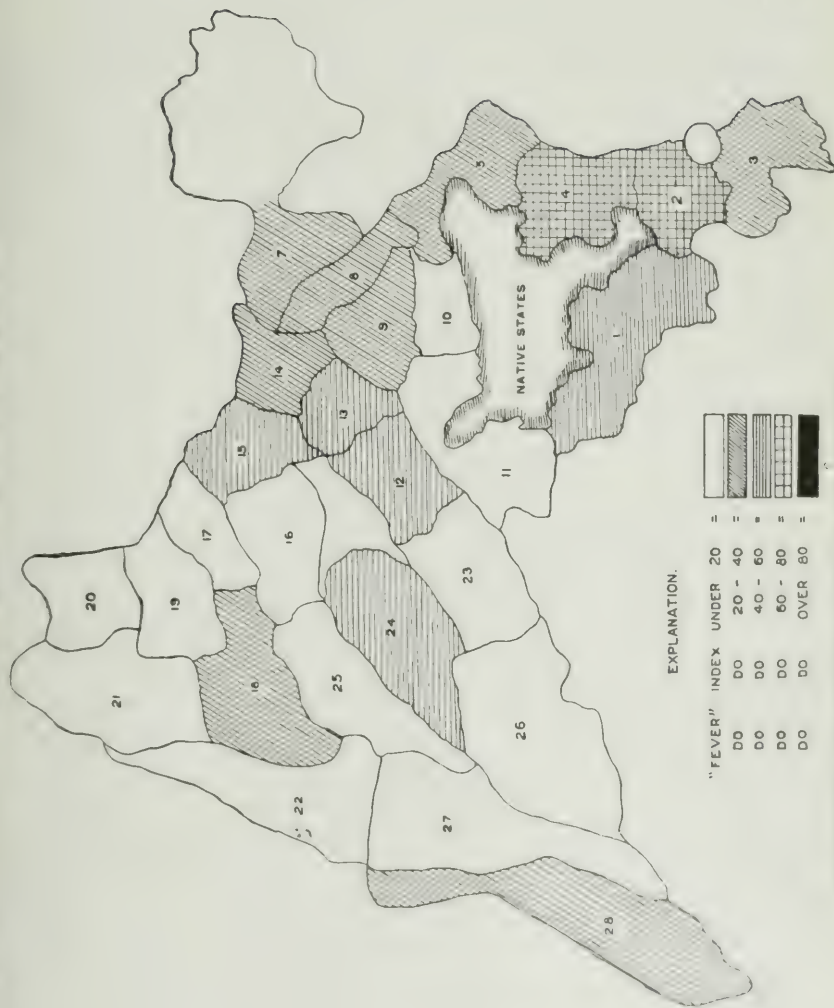


EXPLANATION.

"FEVER" INDEX	UNDER 20	=
DO	20 - 40	=
DO	40 - 60	=
DO	60 - 80	=
DO	OVER 80	=

THE DISTRICTS ARE NUMBERED ACCORDING TO THE NUMERATION SHOWN IN APPENDIX B.



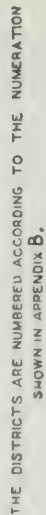


## EXPLANATION.

"FEVER" INDEX	UNDER 20	=
DO	DO	20 - 40
DO	DO	40 - 60
DO	DO	60 - 80
DO	DO	OVER 80

THE DISTRICTS ARE NUMBERED ACCORDING TO THE NUMERATION SHOWN IN APPENDIX B.







taken to verify the presence of relapsing fever by means of the examination of blood films.

In connexion with the clinical aspect of the disease it is desired to emphasise the peculiar liability of relapsing fever to be confused with lobar pneumonia and influenza. So much so is this the case that it is a reasonable assumption that all localised epidemics of these diseases as well as 'pleuro-pneumonia,' and 'septic pneumonia' occurring in the Punjab in the months of May and June, should be regarded, until the contrary be proved, as possibly due to relapsing fever.

Fortunately it is possible, by means of the examination of blood films, to verify at once the presence or absence of relapsing fever, and in no disease is this method of diagnosis of more importance, since by a single injection of salversan or one of its substitutes a prompt and certain cure can usually be obtained.

The prevention of the disease is of even more importance than its cure, but this matter, in the case of the civil population of the Punjab, is beset with great difficulties. It must be presumed that the disease is spread in the Punjab, as elsewhere in India, by the body-louse and that these parasites are universally prevalent more especially in rural areas. To 'de-louse' a civil population of about 20 million people is obviously an impossible task, but in certain areas and under certain conditions some action along these lines is not impossible.

Thus in the case of troops, of the police and of prisoners in jails amongst whom some echo of the present epidemic of relapsing fever in the Punjab must be anticipated to occur, no insuperable difficulties need arise in carrying out this measure.

With these remarks it is proposed to close the present study of relapsing fever, which although restricted in scope has, it is thought, sufficed to throw light on an epidemic disease whose identity has long remained hidden amongst the tangled skein of Punjab 'fevers.'

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# APPENDIX A.

## *The Provincial 'Fever' and Respiratory Indices of Relapsing Fever.*

Year.	'Fever' Index.	Respiratory Index.
1867 ..	24.7	—
1868 ..	44.6	+ 14.1
1869 ..	63.0	+ 8.8
1870 ..	10.8	— 11.8
1871 ..	0.0	—
1872 ..	25.6	— 2.4
1873 ..	17.2	—
1874 ..	21.2	—
1875 ..	7.4	—
1876 ..	12.2	—
1877 ..	22.1	—
1878 ..	53.6	—
1879 ..	14.9	—
1880 ..	0.0	—
1881 ..	0.0	—
1882 ..	21.2	—
1883 ..	3.1	—
1884 ..	20.2	—
1885 ..	27.9	—
1886 ..	27.2	—
1887 ..	15.7	—
1888 ..	30.8	—
1889 ..	26.2	—
1890 ..	20.6	—
1891 ..	40.9	—

APPENDIX A. *contd.*

Year.	'Fever' Index.	Respiratory Index.
1892	0.0	—
1893	16.2	—
1894	15.4	—
1895	22.9	—
1896	14.0	—
1897	23.1	—
1898	28.2	—
1899	8.9	—
1900	33.6	—
1901	34.9	—
1902	9.0	—
1903	7.2	—
1904	3.6	—
1905	0.0	-12.3
1906	38.5	+ 8.5
1907	0.0	- 7.6
1908	11.2	-19.9
1909	0.0	-17.6
1910	13.4	- 9.1
1911	7.9	0.0
1912	3.4	- 7.6
1913	16.9	-13.1
1914	27.6	+10.4
1915	26.2	+ 3.6
1916	12.7	-15.1
1917	3.3	-28.0
1918	17.4	+ 6.8
1919	18.5	+25.0
1920	44.2	+21.8

# APPENDIX B.

*The 'Fever' and Respiratory Indices of each district of the Punjab during the years 1917, 1918, 1919 and 1920.*

District.	1917		1918		1919		1920	
	'Fever'	Respira- tory	'Fever'	Respira- tory	'Fever'	Respira- tory	'Fever'	Respira- tory
1. Hissar ..	23.0	-42.5	32.1	-35.7	45.6	+35.0	47.5	+14.8
2. Rohtak ..	19.5	-24.4	87.4	+23.5	60.3	+38.8	77.4	- 7.3
3. Gurgaon ..	33.8	-15.9	64.4	-34.6	23.3	+36.0	14.9	-11.1
4. Karnal ..	3.8	+16.1	57.2	+72.8	70.5	+74.0	49.5	-15.3
5. Ambala ..	2.9	-18.5	20.2	+12.4	26.7	+126.4	73.0	+119.8
6. Kangra ..	3.1	-46.1	88.0	+ 3.8	31.1	+ 5.8	55.3	+17.0
7. Hoshiarpur ..	nil	-33.6	72.7	+18.4	22.5	-11.0	32.6	-29.7
8. Jullundur ..	3.4	-27.5	nil	+ 4.8	20.5	-15.6	51.1	-20.1
9. Ludhiana ..	0.9	-32.4	nil	-13.4	16.7	+29.1	67.4	+23.1
10. Ferozepur ..	10.1	-36.7	29.7	-25.5	nil	+19.6	23.0	-12.0
11. Lahore ..	9.1	-12.4	34.6	-14.4	44.0	+ 8.7	87.9	+13.0
12. Amritsar ..	6.5	+ 5.2	39.2	+22.0	50.5	+70.0	79.4	+48.7
13. Gurdaspur ..	6.4	- 8.9	46.7	+37.7	20.1	- 7.3	77.7	+24.5
14. Sialkot ..	8.3	-20.7	46.3	+14.5	41.4	+22.4	54.2	nil
15. Gujranwala ..	2.8	-19.1	nil	- 4.4	nil	+57.6	40.6	-14.4
16. Gujrat ..	nil	-19.5	nil	+36.6	2.8	-25.6	nil	+17.5
17. Shahpur ..	21.2	-86.8	nil	-56.5	36.5	+ 4.3	11.3	-27.5
18. Jhelum ..	nil	nil	nil	-15.5	6.2	nil	nil	- 6.5
19. Rawalpindi ..	nil	-49.0	nil	- 8.3	nil	+53.3	nil	-17.2
20. Attock ..	nil	-42.9	nil	+11.3	nil	+51.4	nil	- 7.4
21. Mianwali ..	nil	-27.0	nil	+ 5.2	nil	+186.0	10.2	nil
22. Montgomery ..	10.4	-36.3	nil	-28.1	nil	+118.1	27.9	+34.7
23. Lyallpur ..	24.4	-22.4	47.8	-12.6	49.0	+15.3	58.7	-29.8
24. Jhang ..	2.7	..	nil	-10.0	nil	+34.7	13.8	-39.8
25. Multan ..	1.2	-13.4	nil	-23.3	nil	+26.2	10.2	-12.6
26. Muzaffargarh ..	nil	-76.3	nil	-52.5	nil	-29.0	57.6	nil
27. Dera Ghazi Khan	nil	-30.7	nil	+34.2	29.7	-17.6	5.7	-22.8

## NOTE TO APPENDIX B.

It is not possible to attribute the same significance to the respiratory indices of districts as to the 'fever' indices on account of the fact that the paucity of the figures, more especially in the case of the districts of Mianwali, Muzaffargarh and Dera Ghazi Khan, unduly exaggerate an index formed by means of percentages. In some districts it is also clear that deaths due to respiratory diseases are mainly reported under the head of 'fevers.'

## VISCERAL INFECTIONS DUE TO THE HIGHER FUNGI.

BY

MAJOR F. P. MACKIE, I.M.S.

[Received for publication, August 4, 1921.]

### PULMONARY ASPERGILLOSIS COMPLICATING KALA-AZAR.

*Case I.*—The patient was a British soldier and was admitted to one of the Baghdad hospitals during 1918 with the following history. He had been for 18 months in Mesopotamia and had spent a short time in India before re-drafting.

His illness began with a rigor 18 days before I saw him, and the rigors had continued and were not influenced by quinine. The spleen was noticed to be enlarged a fortnight after the commencement of the disease. His diagnosis on admission had been enteric group fever but blood cultures had proved negative.

*Condition on admission.*—The spleen was much enlarged and tender, reaching to four fingers breadth below the costal margin. Liver slightly enlarged. Diminution of the breath sounds and vocal resonance at the base of the right lung where numerous fine crepitations were heard.

*Progress.*—The disease began on March 13th and the patient died on September 4th, making the course nearly six months.

The above signs persisted and wasting supervened. The lung condition became more pronounced and patches of consolidation were noticed. The cough was troublesome but no sputum was obtained till a few days before death. The spleen continued to increase in size and emaciation eventually became extreme. Subcutaneous hemorrhages, transient patchy edemas and a subcutaneous abscess were other noteworthy signs, which appeared before death.

*Differential diagnosis.*—During the early stages the condition was taken for enteric fever but several blood and faecal examinations were negative.

*Tuberculosis of the lungs.*—This was the diagnosis most confidently made on account of the persistent and progressive signs at the right base. No sputum could be obtained.

*Sub-diaphragmatic abscess.*—Hectic fever, rigors and the signs at the base of the lung and the downward pushing of the liver were signs in favour of this diagnosis. Two blood counts were made with the following result :—

*July 2nd.*—

Red cells	=5,250,000 per cm.	
White cells	=2,031	„ „
Polymorphonuclears	=72·4	per cent.
Small mononuclears	=19·6	„
Large mononuclears	=7·3	„
Eosinophiles	=0·7	„

*August 8th.*—

White cells	=6,500 per cm.	
Polynuclears	=72·0	per cent.
S. Mononuclears	=15·0	„
L. „	=11·0	„
Eosinophiles	=1·5	„
Basophiles	=0·5	„

*Malaria.*—This was suspected on account of the frequent rigors and the splenic enlargement but was excluded by blood examinations and by the negative effect of quinine.

The long continued fever, the wasting, enlargement of the spleen and liver, and the low leucocyte count with high mononuclear figure suggested *kala-azar*. The spleen was punctured early in the disease but *Leishmania* were not found and the patient refused further spleen punctures.

The possibility of the disease being suppurative or tuberculous pyelitis was also considered but though the urine contained albumen from time to time neither cocci nor tubercle bacilli were ever found.

During one of the early attempts at blood culture for the enteric group the bile salt medium on being plated out on McConkey's medium yielded a pure growth of slow growing streptothrix colonies, and on this ground I thought the case to be one of those rare cases of disseminated

nocardiasis which are known to occur and to resemble chronic tuberculosis.

Also I was mindful of the two genuine cases of streptothrix infection which are to be described further on and thought I had found one in this case also.

The source of the streptothrix in this particular case will never be known but a series of puzzling cases which had occurred about this time which were characterised by signs of consolidation and cavitation at the base of the lung, hectic fever rigors and wasting led Lt.-Col. Sprawson and me to describe this group of cases as "Disseminated Nocardiasis" (*vide Indian Medical Gazette*, September 1918, pp. 321 *et seq.*).

Under the influence of this idea I spent several months in the study of material from several cases and in attempting to reproduce the fungus in laboratory animals.

The fact that the fungus was nonpathogenic to these animals and was subsequently found to be occurring as a contamination on plates exposed in the laboratory caused me to modify the attitude I had adopted and to look elsewhere for the cause of this curious symptom complex.

The patient died on September 1th and the autopsy was made within an hour of death.

The most noteworthy changes were in the spleen and the lungs, the former weighed about one pound and was soft and friable. The right lung was toughly adherent to the chest wall especial at the base. On section the bulk of the lower lobe was honeycombed with small abscess cavities ranging in size from a pea to a pin's head. Around these cavities the lung substance was thickened and fibrosed whilst the cavities themselves were occupied by a thick greenish pus. No tubercles could be seen. The left lung was free of adhesions but the lower lobe was in a condition of pneumonic consolidation with the distribution of an aspiration pneumonia.

*Films*—Were made from all the principal organs.

Those from the spleen shewed *Leishmania* in large numbers.

Those from the cavities in the right lung shewed a large amount of a fungus of aspergillus type.

The left lung shewed large numbers of cocci of different types and a small quantity of aspergillus mycelium.

*Cultures*—Were made of all the principal organs and those from the right lung grew an abundance of greenish fungus which quickly overgrew

the medium. A large number of aerial hyphae appeared giving the culture a soft felted appearance and later the whole changed into a dark almost black mat. The fungus was readily cultivated on a variety of media particularly on those containing sugars. In fluid media it formed a thick green pellicle leaving the underlying fluid clear.

*Animal Experiments.*—Pigeon. The sporing fungus on agar was shaken up with normal saline and injected into the muscles of the bird's leg. A month later the bird was killed though it shewed no signs of disease. A nodular thickening of the injected muscle was found and when this was sectioned there was found a local development of the aspergillus mycelium ramifying amongst the muscle fibres. The other organs were not found to be affected.

*Rabbit.*—The material was introduced into the peritoneal cavity and the animal was killed a month later though it shewed no obvious signs of disease. The peritoneum was unchanged but the abdominal lymph glands were found to be enlarged and to exhibit small caseous areas. Films from these areas shewed the presence of abundant aspergillus mycelium and sections revealed the fact that the fungus had invaded the gland substance to a considerable depth around the caseous patches.

*Sections from the human lung* (Plates LXVI and LXVII).—An extensive invasion of the lung tissue was observed which penetrated for a considerable distance around the inflamed areas. The mycelium ramified chiefly in the alveolar interspaces and projected into the alveoli themselves; the lung tissue was in a condition of chronic fibrosis whilst in the neighbourhood of the abscess cavities the tissues were partially liquified and converted into a collection of pus cells, fragments of mycelium and some black pigment granules presumably of fungus origin.

The history of the case and the post mortem findings make it highly probable that the aspergillus infection of the lung was not merely a terminal infection but one of long standing perhaps dating from the very beginning of the clinical symptoms. Whether it was primary, secondary or coincident to the Kala-azar infection it is impossible to say.

#### TWO CASES OF STREPTOTHRIX INFECTION OF THE BRAIN.

*Case II.*—Private E. had been four months in Mesopotamia and previously one month in India. Soon after arriving in Mesopotamia he developed irregular fever with headache and retro-orbital pain. There was no history of any tropical disease except that he gave a history of

534

**EXPLANATION OF PLATE LXVI.**

**Aspergillosis of the lung.**

The portion adjoining an abscess cavity.

(a) Remains of lung alveoli filled with mycelium and purulent debris

(b) Massive invasion of the inter-alveolar septa by the fungus.

**Magnification**— $\times 82$  Std. hæmatoxylin and eosine.

EXPLANATION OF PLATE XXVI.

Aspergillus of the lung.

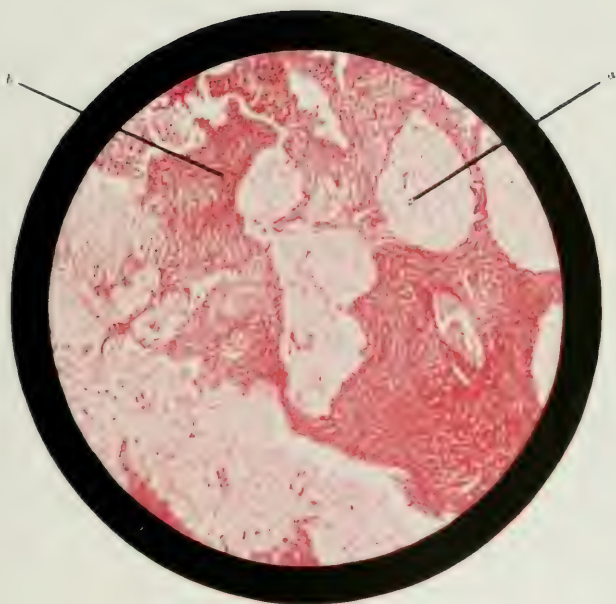
The portion adjoining an abscess cavity.

(a) Remains of lung alveoli filled with mycelium and purulent debris

(b) Massive invasion of the inter-alveolar septa by the fungus.

Magnification =  $\times 82$  Std. haematoxylin and eosine.

PLATE LXVI





EXPLANATION OF PLATE LXVII.

Higher magnification of the above.

Shews a portion of an inter-alveolar septum invaded by the aspergillus fungus which is projecting into the alveolus.

Magnification  $\times 240$ . Hæmatoxylin and eosine

EXPLANATION OF PLATE LXVII.

Magnification— $\times 340$ . Hematoxylin and eosine  
fungus which is projecting into the alveolus.  
Shows a portion of an inter-alveolar septum invaded by the aspergillus  
Higher magnification of the above.

PLATE LXVII

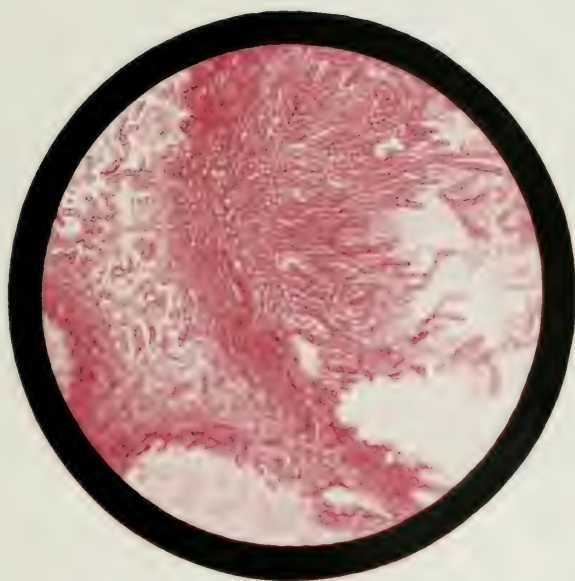
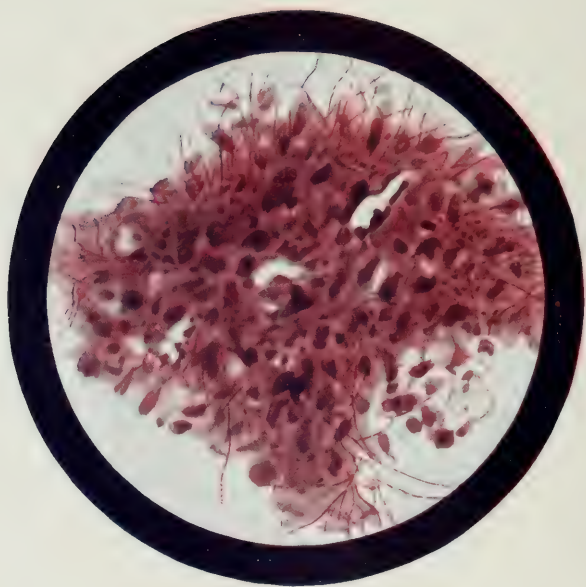






PLATE LXVIII.



EXPLANATION OF PLATE LXVIII.

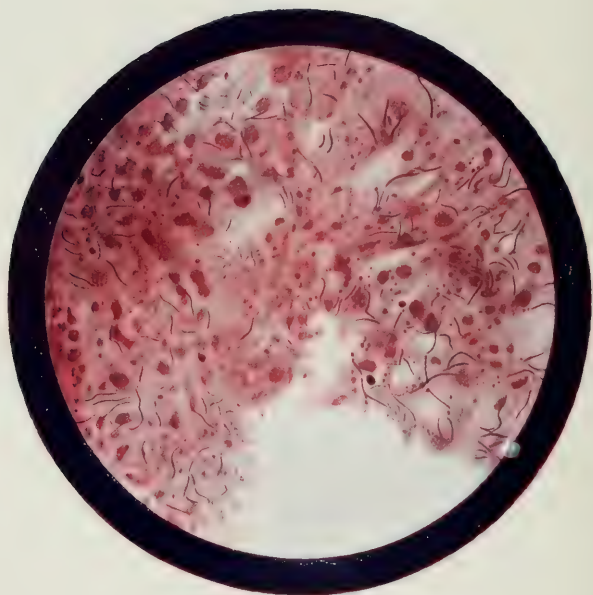
A transverse section of the cerebellum showing the  
arborescent structure of the arbor vitae.

EXPLANATION OF PLATE LXVIII.

A granule of pus from the cerebral abscess shewing streptothrix mycelium.



PLATE LXIX



EXPLANATION OF PLATE LXIX.

Section from the brain abscess showing the streptothrix ramifying in  
the margin of the cavity.

EXPLANATION OF PLATE LXIX.

Section from the brain abscess shewing the streptothrix ramifying in the margin of the cavity.

passing dysentery-like stools and during his stay in hospital *Entamoeba Histolytica* were found in his faeces. The only marked clinical feature was drowsiness and mental hebetude. No physical signs were found in any of the viscera. He was in hospital for three weeks and during that time had irregular fever which during the last week oscillated between 102 and 104. He became increasingly drowsy and died in a comatose state.

The usual pathological examinations were made during life without throwing any light on the cause of his symptoms.

The total leucocyte count was 11,000 per cm. of which 65 per cent. were polymorphs, 21·5 per cent small mononuclears, 8 per cent large mononuclears, 4·5 per cent transitional and 1 per cent were eosinophiles.

The post mortem examination was made a few hours after death.

All the organs except the brain were healthy.

The pia-arachnoid was congested and lustreless and the membranes were adherent by recent but not fresh lymph especially at the sulci and along the fissure of Sylvius.

There were several soft bulging areas in the cortex which were found on section to be abscess cavities containing some glutinous greenish pus. One cavity the size of a chestnut was situated near the anterior end of the caudate nucleus and had burst into the lateral ventricle both of which contained thin semipurulent fluid. No granules were seen in the pus such as are met with in actinomycosis.

On microscopic examination of the pus a large amount of streptothrix mycelium was seen ramifying in the pus. (*Vide* Plate LXVIII.) The fungus grew well on most laboratory media especially those containing sugars such as saccharose and glucose but the cultures were contaminated with a saprophytic bacillus present in the abscess cavities.

In later cultures the fungus broke up into small segments and finally into minute coccoid granules. Animal experiment was attempted but the guinea-pig died a few days later of secondary bacterial infection.

The pus was carefully searched but no entamoebae were found. Sections of the wall of the brain abscess (*Vide* Plate LXIX) showed chronic inflammatory changes with degeneration and destruction of the cortical cells. The walls of the abscess were composed of partially liquified tissue in which was ramifying a tangled mass of streptothrix

mycelium and this penetrated for some distance into the cerebral substance around the cavity.

*Case III.*—Private F. was in hospital for six weeks with recurrent fever of an intermittent type.

He had been 28 months in Mesopotamia and previously 7 years in India. He had suffered recently from headaches and pain in the eyes and had had bouts of fever from time to time. Previous to admission he had had dysentery and more recently benign tertian malaria and a nonvirulent type of diphtheria. In the early stages of his illness in hospital he shewed a high leucocytosis which was

Total leucocyte count	47,000 per cm.
Polynuclears	92.5 per cent.
Small mononuclears	5.0 per cent.
Large       ,,	2.5 per cent.

His temperature rose rapidly a few days before death and he became comatose and died from what was believed to be heatstroke.

At the autopsy all the abdominal and thoracic viscera were found to be normal.

There was an abscess the size of a chestnut occupying the junction of the crura cerebri and the pons.

The cavity contained thick greenish pus which on microscopic examination shewed abundant streptothrix mycelium. Plate LXX depicts the delicate type of branching mycelium found in this case which was similar to that found in Case II. No amœbæ were found in the pus.

Sections of the wall of the cavity shewed appearances very similar to those seen in Plate LXIX from the last case. The mycelium was growing in the abscess wall and ramifying for some distance into the inflamed cerebral tissue.

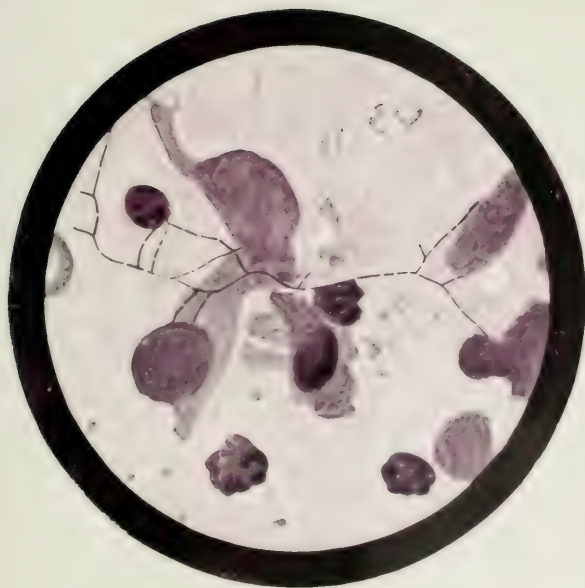
**EXPLANATION OF PLATE LXX.**

**Mycelial fragment from the pontine abscess in Case II.**

**Plates III, IV and V, all drawn at a magnification of 820 diameters  
and all stained by Gram's method.**

EXPLANATION OF PLATE LXX.

Myoeial fragment from the pontine spaces in Case II.  
Plates III, IV and V, all drawn at magnification of 820 diameters  
and all stained by Gram's method.





THE CORRELATION BETWEEN THE CHEMICAL  
COMPOSITION OF ANTHELMINTICS AND  
THEIR THERAPEUTIC VALUES IN  
CONNECTION WITH THE  
HOOKWORM INQUIRY  
IN THE MADRAS  
PRESIDENCY

BY

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[Received for publication, August 13, 1921.]

XV SALICYLIC ACID AND ITS DERIVATIVES

HERMANN, of Mons, in Belgium, is reported to have treated some cases of ankylostomiasis with a mixture containing oil of wintergreen 2 grammes-, chloroform 3 grammes-, and castor oil 40 grammes, but no mention is made of the results obtained from this mode of treatment.<sup>1</sup>

*Oleum Gaultheriae.*

Oil of wintergreen is distilled from the leaves of *Gaultheria procumbens*, Linn., or from the bark of *Betula lenta*, Linn. It is a colourless or almost colourless liquid, with a strong characteristic odour and a pungent taste; specific gravity: 1.180 to 1.187; boiling point: 218° to 221° C.; optical rotation at 25° C.: 0° to 1°; refractive index: 1.537 to

1.539; soluble in 6 parts of alcohol at 70 per cent. It contains, when pure, about 99 per cent of methyl salicylate.

The oil we used was found to conform to the reactions and tests given in the Pharmacopœia. It contained 98.5 per cent of methyl salicylate, and traces of an aldehyde.

As a test treatment the drug was administered together with castor oil in gum acacia emulsion in two portions at half an hour's interval. The alimentary canal had previously been prepared by purgation with magnesium sulphate.

TABLE I.

*Number of hookworms removed by one 'test treatment' of Oleum Gaultheriae.*

Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A 'TEST TREATMENT.'		
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
3 to 20 minims ..	19	Test treatment ..	1	18	19	6.2	9.9	9.6
		Subsequent treatments*	15	163	178			
		Total hookworms	16	181	197			
22 to 40 Do. ..	17	Test treatment ..	1	32	33	4.3	20.0	17.9
		Subsequent treatments.	22	129	151			
		Total hookworms..	23	161	184			
42 to 58 Do. ..	15	Test treatment ..	1	93	94	5.0	32.7	30.9
		Subsequent treatments.	19	191	210			
		Total hookworms ..	20	284	304			
60 Do. ..	20	Test treatment ..	4	159	163	13.3	50.1	46.5
		Subsequent treatments.	29	158	187			
		Total hookworms..	33	317	350			

\* Invariably 60 grains thymol.

As may be gathered from Table I oil of wintergreen has well marked anthelmintic properties. Its action is vermicide for *most* of the worms are expelled dead and in a state of flaccidity. It is, however, ineffective

for the removal of such non-bursated nematodes as *Ascaris* and *Trichiuris*: thirteen of our cases were infected with ascaris and nine with trichiuris, but none of them passed worms.

Oil of wintergreen is a safe drug and, with the exception of two cases who complained of giddiness, the treatment was well borne. No after effects were noticed.

#### METHYLIS SALICYLAS.

The mode of treatment was exactly the same as that previously adopted when working with the oil. As was to be expected from the composition of oil of wintergreen the anthelmintic value of pure methyl salicylate is practically the same as that of the oil.

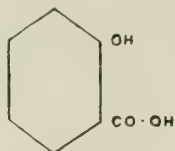
TABLE II.

*Number of hookworms removed by one 'test treatment' of  
Methylis salicylas.*

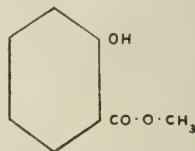
Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.				PERCENTAGE OF HOOKWORMS REMOVED WITH A 'TEST TREATMENT.'			
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.		A. duodenale.	N. americanus.	A. duodenale and N. americanus.	
30 minims	15	{ Test treatment ..	4	41	45		16.6	18.0	18.6	
		{ Subsequent treatment.	20	176	196					
		{ Total hookworms..	24	217	241					
60 lbs.	19	{ Test treatment ..	0	291	291		0.0	51.4	50.8	
		{ Subsequent treatment.	6	275	281					
		{ Total hookworms..	6	566	572					

The following points may, however, be noted :- 1. None of twelve trichiuris infected cases passed worms, but two of fifteen ascaris infected cases did : 2. *all* the hookworms expelled were dead and flaccid : 3. the total absence of toxic symptoms shows that the aldehydic content of the oil was probably responsible for the giddiness recorded.

Methyl salicylate is formed by substituting methyl for hydrogen in the carboxyl group of salicylic acid.

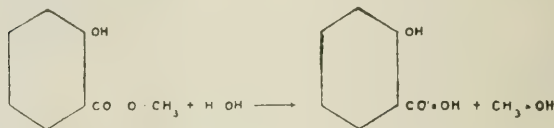


SALICYLIC ACID



METHYL SALICYLATE

Pharmacologically methyl salicylate belongs to one of the two groups of substances known as 'partial salols':—a group of esters in which an active (aromatic) acid is esterified with an inert hydroxylic substance (alcohol), and which on hydrolysis liberate the alcohol and the acid. Thus methyl salicylate breaks down in the intestine with the liberation of salicylic acid, and its physiological action is that of the acid minus its toxicity.



METHYL SALICYLATE

SALICYLIC ACID  
(active)METHYL ALCOHOL  
(inert)

The question which naturally arises here is whether the anthelmintic properties of methyl salicylate are due to the liberation of free salicylic acid in the duodenum or whether the ester itself exerts a specific anthelmintic action in the unhydrolyzed state.

#### ACIDUM SALICYLICUM.

Pure salicylic acid was administered in two portions at an hour's interval on an empty stomach following upon an overnight purgation with magnesium sulphate. Two hours after the administration of the second portion of the drug the bowels were washed with magnesium sulphate. This mode of treatment was also followed later when working with salol, betol, and aspirin.

No toxic symptoms developed, nor were any after effects noticed.

TABLE III.

Number of hookworms removed by one test treatment of *Acidum salicylicum*.

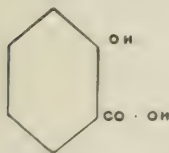
Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.				PERCENTAGE OF HOOKWORMS REMOVED WITH A TEST TREATMENT.			
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.		A. duodenale.	N. americanus.	A. duodenale and N. americanus.	
30 grains	15	Test treatment ..	0	2	2		0.0	1.2	1.0	
		Subsequent treatments.	6	193	199					
		Total hookworms	6	195	201					

The results prove that salicylic acid is ineffective against hookworms. Of nine cases who were found infected with ascaris one passed worms, but the three trichiuris cases showed no response to the drug.

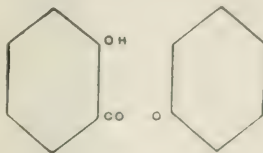
It follows from this that methyl salicylate exerts a specific anthelmintic action in the unhydrolyzed state. And the striking behaviour of salol gives full support to this conclusion.

#### PHENYLIS SALICYLAS.

Salol is obtained from salicylic acid by substituting phenyl for hydrogen in the carboxyl group.

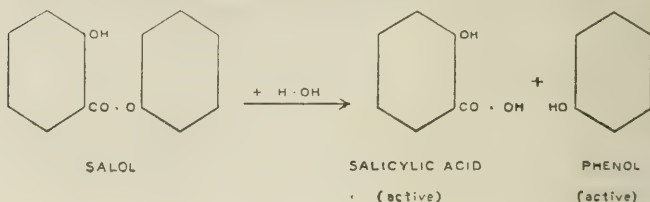


SALICYLIC ACID.



SALOL

Salol is, therefore, an ester resulting from the combination of two physiologically active substances : phenol and salicylic acid. The ester on hydrolysis in the intestine thus liberates two powerful antiseptics.



All our 37 cases stood the treatment remarkably well, and neither salicylic acid intoxication nor phenol poisoning were produced.

TABLE IV.

*Number of hookworms removed by one 'test treatment' of Phenylis Salicylas (Salol).*

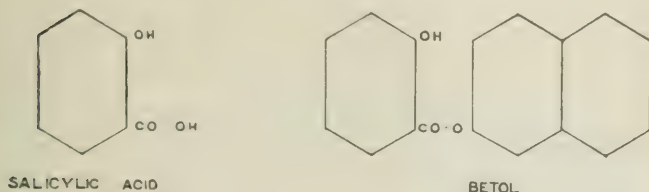
Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A 'TEST TREATMENT.'			
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.	
10 to 20 grains ..	8	Test treatment ..	0	0	0	0.0	0.0	0.0	
		Subsequent treat- ments.	9	33	42				
		Total hook worms.	9	33	42				
25 to 40 Do. ..	7	Test treatment ..	0	0	0	0.0	0.0	0.0	
		Subsequent treat- ments.	14	76	90				
		Total hookworms..	14	76	90				
45 to 55 Do.	7	Test treatment ..	0	0	0	0.0	0.0	0.0	
		Subsequent treat- ments.	1	32	33				
		Total hookworms..	1	32	33				
60 Do. ..	15	Test treatment ..	0	0	0	0.0	0.0	0.0	
		Subsequent treat- ments.	17	314	331				
		Total hookworms..	17	314	331				

Table IV calls for no explanation. If we add that, of eleven cases who harboured ascaris and seven who harboured trichiuris none passed worms, the total absence of anthelmintic properties in salol is evident.

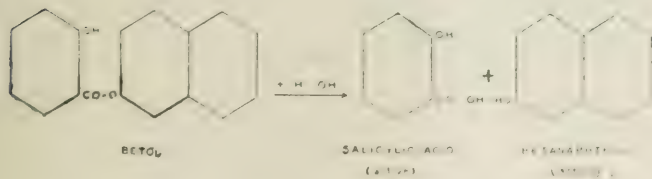
Castellvi had already noted the inefficiency of salol in the treatment of ankylostomiasis.<sup>3</sup>

### BETOL.

If we combine betanaphthol with salicylic acid through the agency of phosphorus oxychloride we obtain the salicylic ester of betanaphthol known as 'betol.' This compound may be looked upon as formed either (1) by substituting naphthyl for hydrogen in the carboxyl group of salicylic acid, or (2) by substituting salicyl for hydrogen in the hydroxyl radical of betanaphthol, or again (3) by substituting naphthyl for phenyl in the molecule of salol.



Pharmacologically betol is a true salol which on hydrolysis in the intestine breaks down with the liberation of free salicylic acid and free betanaphthol.



Betol proved ineffective against hookworms—20 cases—ascaris—1 cases, and trichiuris—8 cases.

TABLE V.

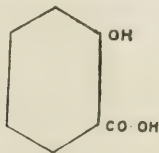
*Number of hookworms removed by one "test treatment" of Betol.*

Test treatment.	Number of cases treated.		HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A "TEST TREATMENT."		
			A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
30 grains	16	Test treatment ..	0	0	0	0.0	0.0	0.0
		Subsequent treatments.	27	487	514			
		Total hookworms..	27	487	514			
60 Do.	4	Test treatment ..	0	2	2	0.0	0.4	0.4
		Subsequent treatments.	9	428	437			
		Total hookworms..	9	430	439			

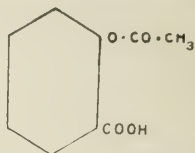
As 30 and 60 grains doses of betol must have liberated 16 and 32 grains, respectively, of free betanaphthol in the duodenum, the absence of vermicial and vermifugal properties leads us to admit that :—1. the esterification of betanaphthol destroys the anthelmintic action of that substance ; 2. the 'salol principle' cannot be extended to anthelmintics.

## ACIDUM ACETYLSALICYLICUM.

Salicylic acid, owing to the presence of the hydroxyl as well as the carboxyl groups, can play the part of both phenol and acid. Thus when acetic anhydride or acetyl chloride reacts with salicylic acid at high temperatures acetyl salicylic acid, or aspirin, is formed by substitution of acetyl for the hydrogen in the phenolic hydroxyl of the acid.

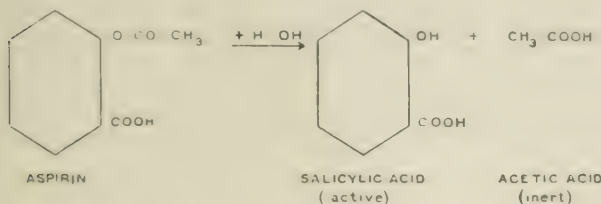


SALICYLIC ACID



ASPIRIN

Aspirin is a 'partial salol' of the second type:—an ester in which an active hydroxyl compound (alcohol or phenol) is esterified by an inactive acid, and whose action therefore resembles that of the alcohol or phenol, the sodium salt of the acid being inert.



We already know that salicylic acid has no anthelmintic value. That aspirin has no specific anthelmintic properties may be seen from Table VI.

TABLE VI.

*Number of hookworms removed by one 'test treatment' of Acidum Acetylsalicylicum (Aspirin).*

Test treatment.	Number of cases treated.	HOOKWORMS REMOVED.			PERCENTAGE OF HOOKWORMS REMOVED WITH A 'TEST TREATMENT.'		
		A. duodenale.	N. americanus.	A. duodenale and N. americanus.	A. duodenale.	N. americanus.	A. duodenale and N. americanus.
30 grains	15	Test treatment ..	0	0	0	0	0
		Subsequent treatments.	36	231	267	0	0
		Total hookworms..	36	231	267	0	0

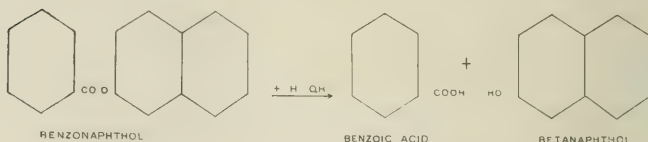
Three of the cases were found infected with ascaris and eight with trichiuris, but none expelled worms.

#### BENZONAPHTHOL, THYMOTAL, DUCIAL.

Though these substances are not derivatives of salicylic acid, we think their properties ought to be discussed here for (1) they are phenols

converted into esters or carbonates according to the 'salol' principle,' and (2) they have been either used or recommended for the treatment of ankylostomiasis.

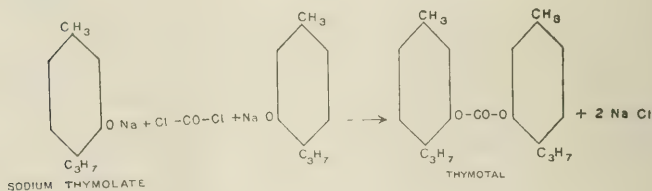
Benzonaphthol is derived from betanaphthol by substituting benzoyl for hydrogen of the hydroxyl group. It is hydrolysed in the organism to benzoic acid and betanaphthol.



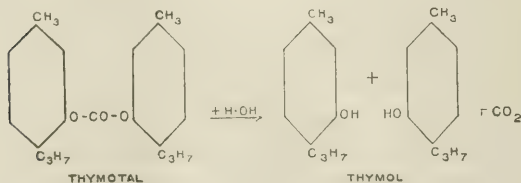
Castellvi used benzonaphthol for the treatment of ankylostomiasis and reported that the drug 'appeared useless and had no response in the patients.'

Our conclusion is, therefore, that with the introduction of the benzoyl group and the disappearance of its phenolic character betanaphthol has lost its vermifugal properties: a conclusion supported by the behaviour of betol in our hands.

Thymol carbonate, known as 'thymotal' or 'thymol urethane,' is prepared by the action of carbonyl chloride on an alkaline solution of thymol



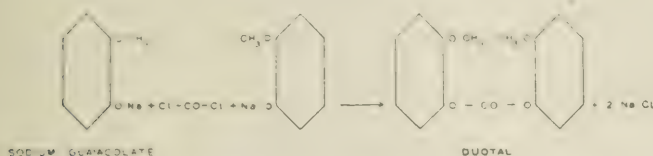
and on hydrolysis.



It was at first claimed that this substance is as effective as thymol against anchylostoma<sup>1</sup>; but Calmette and Breton declared it to be useless,<sup>2</sup> and Schüffner who treated 96 cases with thymol in 5—25 grammes dosages found that only 2.4 per cent of the hookworms were expelled.<sup>3</sup>

These results point again to the loss of anthelmintic properties following upon the suppression of phenolic groups, and clearly exhibit that the vermicial value depends upon a specific action of the substance in the unhydrolysed state.

The action of carbonyl chloride on an alkaline solution of guaiacol leads to the formation of a carbonate known as 'duotal.'



Dock and Bass<sup>4</sup> speak of guaiacol carbonate as having been recommended for the treatment of ankylostomiasis; but we could find no other mention of the drug in the literature. We need not wonder at this for, in the light of our work, the chemical constitution of duotal spells failure.

TABLE VII.

*Comparative Summary of Results.*

Drug.	Dosage.	Number of cases.	Total Hookworm content.	Hookworms removed with a test treatment.	Percentage of hookworms removed with a test treatment.
Salicylic acid ..	30 grains	15	201	2	1.0
Acetyl salicylic (Aspirin) ..	30 "	15	267	0	0.0
Methyl salicylate ..	30 minims	15	241	4	1.6
Salol ..	60 grains	15	331	0	0.0
Betol ..	30 "	16	514	0	0.0
Betanaphthol ..	10 "	22	1248	293	23.4
Betol ..	60 "	4	437	2	0.4
Betanaphthol ..	30 "	32	1131	810	71.2
Thymol <sup>5</sup> ..	5-25 grammes	96	4824	113	2.4
Thymol ..	grammes	374	13423	12784	95.8

## CONCLUSIONS.

1. Salicylic acid has no anthelmintic properties.
  - (a) Esterification with acetic acid leads to the formation of a compound with no anthelmintic value.
  - (b) Esterification with either phenol or betanaphthol leads to the formation of compounds with no anthelmintic value.
  - (c) Esterification with methyl alcohol results in the formation of a compound with well-marked vermicial properties.
2. Esterification of either thymol or betanaphthol results in the loss of the vermicial and vermifugal properties of those two very potent anthelmintics.
3. Anthelmintic action is specific.
4. The so-called 'salol principle' cannot be extended to anthelmintics.

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# DELAYED CURES IN THE TREATMENT OF HOOKWORM INFECTION.

BY

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(Received for publication, August 18, 1921.)

IN the course of my inquiry into the treatment of the Hookworm Infection I came across infected cases who passed no worms after the administration of the anthelmintic and continued being infected as shown by the presence of ova in their stools. As seen in Table I, some of the cases received three, four, and even five treatments without any worms being removed.

TABLE I.

*Hookworm infected cases who showed no worm removal in spite of repeated treatments.*

Case number.	1st Treatment.		2nd Treatment.		3rd Treatment.		4th Treatment.		5th Treatment.	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
5561 ..	0	+	1	+	0	+	0	+	0	..
6321 ..	0	+	0	+	0	—	..	..	..	..
6473 ..	0	+	0	+	0	—	..	..	..	..
6563 ..	1	+	57	+	0	+	0	+	0	—
6591 ..	3	+	1	+	0	+	0	+	0	—
6605 ..	0	+	0	+	0	—	..	..	..	..
6921 ..	0	+	0	+	0	+	0	+	0	—
6832 ..	1	+	5	+	0	+	0	+	0	—
6982 ..	48	—	22	+	0	+	0	+	0	—
7326 ..	16	+	0	+	0	+	0	—	..	..

(a)=Number of hookworms removed after treatment.

(b)=Ova Infection twelve days after treatment.

TABLE I.—*contd.*

Case number.		1st Treatment.		2nd Treatment.		3rd Treatment.		4th Treatment.		5th Treatment.	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
7335	..	6	+	0	+	0	+	0	—	..	..
7467	..	84	+	0	+	0	+	0	+	0	—
7502	..	5	+	0	+	0	+	0	—	..	..
7803	..	109	+	0	+	0	+	0	—	..	..
6635	..	2	+	18	+	0	+	0	+	0	+
7089	..	1	+	6	+	0	+	0	+	0	+
7208	..	4	+	2	+	0	+	0	+	0	+
7221	..	0	+	0	+	0	+	0	+	..	..

(a)=Number of hookworms removed after treatment.

(b)=Ova Infection twelve days after treatment.

Thymol in doses of 60 grains was generally used, but was often alternated with 50 grains betanaphthol. The drug was administered in two portions at an hour's interval. The treatment was preceded and followed by a dose of Epsom salts and no food was allowed until the bowels had moved. The stools passed after treatment were collected, washed, and examined for five days. Half a c.c. of fecal matter was thoroughly searched for hookworm ova, by the centrifuge method, twelve or more days after the treatment, and if found infected the case was immediately given another treatment.

Most of the cases did, however, get cured in the course of time. As an explanation it occurred to me that an anthelmintic may react on the parasite in various ways. Some of the worms may be killed outright, and some may be sufficiently stunned to be washed away by a purgative. Others, however, may be so slightly affected as to revive and fix themselves in the lumen of a lower portion of the intestine where they will carry on their ovulating function. These worms are thus beyond the reach of the anthelmintic and safe from its baneful effects whatever be the number of treatments; but removed from their elective site and placed in uncongenial surroundings they must soon die a natural death. If this surmise is correct, some of the cases that pass no hookworms on treatment and yet show infection under the microscope twelve days later, ought to get cured without any further treatment.

All such cases were, therefore, kept under observation. The diagnosis of the cure was made more rigid, being based on four

examinations of half a c.c. of stool two consecutive days of two consecutive weeks. Examinations were repeated for all cases at intervals of about a fortnight. The results are recorded in Tables II, III, IV, and V.

TABLE II.

*Cases found infected on the twelfth day after the last treatment and showing 'delayed cure.'*

Case number.	HOOKWORMS REMOVED AT EACH TREATMENT.					Time of cure after treatment.	Time when last examined.
	1st	2nd	3rd	4th	5th		
6296	2	0	0	..	..	46th day	16th day
7878	0	7	0	0	..	54th ..	247th ..
8047	0	5	29	0	0	36th ..	217th ..
8186	16	0	..	..	..	44th ..	224th ..
8216	0	93	0	0	..	30th ..	198th ..
8244	0	132	1	2	..	42nd ..	183rd ..
8362	0	45	5	..	..	45th ..	122nd ..
8576	0	7	0	..	..	67th ..	127th ..
8587	0	23	0	..	..	44th ..	128th ..
8639	0	0	..	..	..	38th ..	124th ..
8795	4	12	0	0	..	61st ..	95th ..
8850	0	0	..	..	..	21st ..	75th ..
8860	2	58	1	1	..	37th ..	37th ..
8901	0	7	0	..	..	74th ..	74th ..

The cases shown in Table II, though still infected after the last treatment, were found cured without any further treatment after a period varying from three to ten weeks and have remained so to this day.

TABLE III.

*Cases who showed 'delayed cure' and left 'not cured.'*

Case number	HOOKWORMS REMOVED AT EACH TREATMENT.					Time of cure after treatment.	Time when last examined.
	1st	2nd	3rd	4th	5th		
7827	11	0	3	1	0	28th day	67th day
8070	1	0	..	..	..	44th ..	44th ..
8109	0	186	4	1	..	45th ..	45th ..
8211	0	1	0	0	..	29th ..	29th ..
8223	0	21	0	0	0	36th ..	153th ..
8496	0	113	1	..	..	40th ..	60th ..
8496	0	88	0	..	..	42nd ..	60th ..
8599	0	117	0	..	..	40th ..	66th ..
8596	0	20	0	..	..	44th ..	60th ..
8594	0	3	0	..	..	40th ..	60th ..
8611	0	1	0	..	..	39th ..	60th ..
8643	0	0	..	..	..	36th ..	60th ..

TABLE IV.

*Cases who showed 'delayed cure' followed by infection.*

Case number.	HOOKWORMS REMOVED AT EACH TREATMENT.				Time of cure after treatment.	Time of 're-infection.'
	1st	2nd	3rd	4th		
7797 ..	0	4	2	0	38th day	212th day
8017 ..	0	16	0	0	43rd ..	126th ..
8144 ..	0	45	2	..	72nd ..	154th ..
8222 ..	0	12	0	..	42nd ..	128th ..
8407 ..	49	4	0	..	43rd ..	74th ..
8554 ..	16	85	0	..	55th ..	134th ..

In Table IV are recorded six cured cases who became subsequently reinfected. This Table cannot, however, be taken as an index of reinfection, for some of the convicts who left the Jail cured—Table III—might have been reinfected.

TABLE V.

*Cases who did not show 'delayed cure.'*

Case number.	HOOKWORMS REMOVED AT EACH TREATMENT.				Time when last examined.
	1st	2nd	3rd	4th	
8056 .. ..	0	23	0	0	214th day
8235 .. ..	0	144	0	..	90th ..
8344 .. ..	3	20	3	..	140th ..
8387 .. ..	18	4	0	..	55th ..
8583 .. ..	1	21	0	..	131st ..
8585 .. ..	1	80	0	..	118th ..
8680 .. ..	8	6	0	..	99th ..
8700 .. ..	9	0	0	..	94th ..
8703 .. ..	0	4	0	..	102nd ..
8708 .. ..	1	1	0	..	102nd ..
8861 .. ..	0	1	0	..	55th ..

The cases shown in Table V have not yet been found cured.

In the light of the above results it would seem that repeated treatments are not absolutely necessary, and that all that is required is an effective first treatment. An attempt was then made to effect cures after one single treatment with an anthelmintic in (1) optimum, and (2) inadequate dosages. Betanaphthol was given to two series of cases in doses of 50 and 30 grains in a single portion, without any purgative. The convicts were taken up for treatment seven weeks after their incarceration, in order to allow a possible pre-jail infection to develop in the intestine and not vitiate the results of subsequent microscopic examination. Two positive ova findings in the stools on two consecutive days preceding the treatment day were taken as an index of hookworm infection. Microscopical examinations after treatment were carried out as detailed above for cases in Table II.

TABLE VI.

*'Immediate' and 'delayed' cures after one betanaphthol treatment.*

Treatment.	Number of cases treated.	CURES.		PERCENTAGE OF CURES.		TIME TAKEN FOR DELAYED CURE.	
		Imme- diate.	Delayed.	Imme- diate.	Total.	Mini- mum.	Maxi- mum.
BETANAPHTHOL—							
50 grains ..	30	22	3	73.3	86.3	36 days	62 days
30 „ ..	52	23	7	44.2	57.7	32 „	68 „

Table VI shows (1) the percentage of cures immediately following upon treatment, (2) the percentage of delayed cures, and (3) the minimum and maximum number of days required for the delayed cure. Results with 30 grains of betanaphthol show that inadequate dosages act in much the same way as optimum dosages in bringing about delayed cures.

#### CONCLUSIONS.

1. In addition to its immediate vermifugal action, an anthelmintic may also have a remote action on the worms.
2. This remote action brings about 'delayed cures.'
3. Except in proved cases of reinfection, a treatment which is known to be efficient need not be followed by any other.

# THE DEMONSTRATION OF THE CAPSULES OF THE PNEUMOCOCCUS BY A MODIFICATION OF BENIAN'S RELIEF STAIN.

BY

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*On special duty under the Indian Research Fund Association.*

[Received for publication, September 15, 1921.]

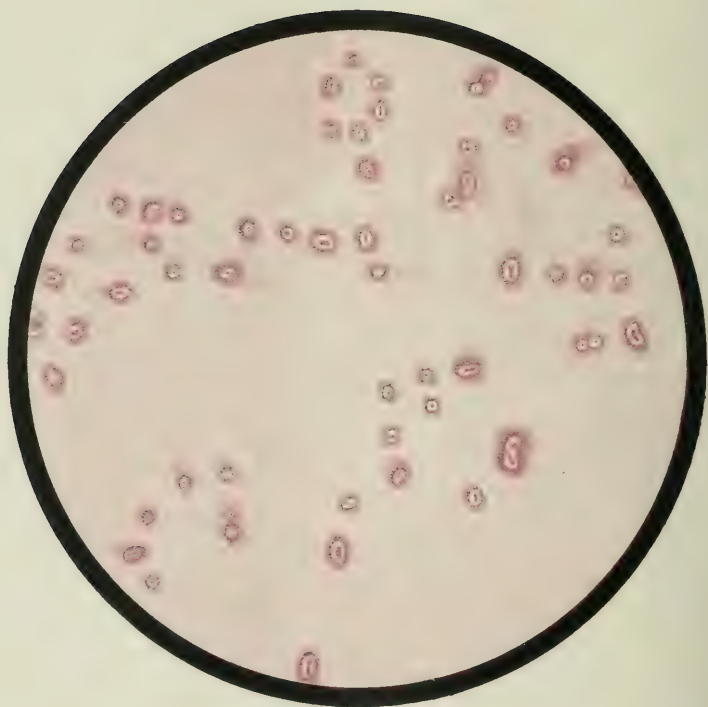
THE presence of a capsule is one of the best means of differentiating pneumococci from allied organisms, but none of the methods of staining capsules gives good results under all conditions. The method which, hitherto, I have found to be the simplest and best is that of Hiss, but even this fails to demonstrate capsules after a freshly isolated organism has been subcultured for a few times, sometimes even after the first subculture. It has also failed on many occasions to demonstrate capsules in the peritoneal fluid and heart's blood of rabbits and guinea-pigs which have died of a pneumococcal septicemia, although the material examined contained abundant pneumococci. This is more often the case when old stock cultures are injected into animals for the purpose of restoring virulence than when comparatively recently isolated cultures are used, or again when very heavy doses of the live organisms have been inoculated.

Stirt in 'Practical Bacteriology Blood Work and Animal Parasitology,' 1915 Edition, p. 37, mentions that 'The India ink method of staining gives good results for capsules,' and in the *British Journal of Experimental Pathology*, 1920, 1, 127, an India ink relief stain has been described in detail.

The method described below is based on the same principle, but gives more constant results than the India ink method; its value in the study of the pneumococci came to my notice early last year during the



PLATE LXXI.



examination of a number of specimens of pus obtained post-mortem from the accessory respiratory sinuses of cases of influenza.

The method is as follows :

1. Make a thin smear of the material to be examined.
2. Fix by heat and allow to cool.
3. Pour a few drops of 2 per cent aqueous Congo red over the slide.
4. Stain for about one minute.
5. Drain off excess of stain and allow the film to dry in air.  
(If a thinner film of stain is desired, it may be made by drawing a drop of Congo red over the slide as in making an ordinary blood film.)
6. Treat the film, when dry, with 1 per cent HCl in 96 per cent alcohol for a few seconds.
7. Allow to dry in air.
8. Counterstain with 0.5 per cent aqueous methyl violet for about one minute.
9. Wash off excess of stain with tap water.
10. Blot or allow to dry in air.
11. Examine with the oil immersion lens.

Before treatment with acid alcohol the organisms are seen poorly, as red cocci surrounded by a faintly stained (pink) capsule on a red background. After treatment the back-ground becomes blue, the organisms themselves appearing as pale blue shadowy figures.

To bring out the organisms clearly a method of counterstaining is used. Of the common stains I have found methyl violet and gentian violet to be the most satisfactory—methylene blue stains the organisms rather poorly.

The value of the method lies in its simplicity and the absolute certainty with which capsules can be demonstrated. It has never failed to show the capsules in the peritoneal fluid or heart's blood of inoculated animals, and in subculture is usually successful when Hiss's method fails. There is no heating of the film during the staining process, and presumably therefore little alteration in the size and shape of the capsules.

The coloured Plate LXXI illustrates the appearance of a first subculture from the peritoneal fluid of a rabbit inoculated with a stock stain of pneumococcus Type 1. The lavender colour of the back-ground, the violet of the organisms and the stain adhering to the capsules are well shown.

## PRELIMINARY NOTE ON SEPTIC TANK LATRINES IN RELATION TO HOOKWORM DISEASE.

BY

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[Received for publication, July 22, 1921.]

It has long been an article of sanitary faith that the installation and use of septic tank latrines in a given area would stop the direct spread of hookworm infection from one person to another in that area, and it has been suggested that the value of the effluent as manure might be a dividend on the capital expenditure involved in erection.

The reasons for the belief are founded on the known facts that ankylostome ova have a higher specific gravity than water and sink therein if the water is still, and that they cannot develop in the absence of oxygen; when deprived of this they die.

It is perfectly true that these ova can experimentally and in practice be killed by drowning, but this process may take several weeks. They remain, as was pointed out by Looss, capable of development even when they have been shut off from communication with the air for some time.

The water in a septic tank, and especially in an overworked septic tank, is by no means at rest. In the liquifying chamber great bubbles of gas tear up from the bottom carrying with them fragments of decomposing faecal material. When this occurs near the lip of the purifying chamber these fragments may be, and often are, carried over and similar reactions are set up in the second chamber: so that in an overworked tank partially decomposed faecal material may even escape on to the filter beds.

When such conditions are pictured it is obvious that there is at least a possibility of the escape of living ankylostome ova, and a remoter chance of their development into larvae. With the permission of Dr. Bentley, Sanitary Commissioner with the Government of Bengal, Mr. Dallis, the septic tank engineer to the Department, has brought me many samples of septic tank effluent taken at random from various jute mills and other factories in and around Calcutta. As a rule, two samples were taken at the same time from each tank, one of the filtered effluent and the other of the unfiltered. Glass stoppered bottles holding about ten ounces each were used for collection. The technique of the examination was the same in all cases. The samples collected in the early morning were brought to my laboratory at about 10 A.M. The contents of each bottle were decanted into separate glass vessels and allowed to settle for twenty-four hours. At the end of this period all but about 10 c.c. of the liquid was syphoned off. The remainder was then centrifugalized. From the bottom of the centrifuge tube about 1 c.c. was sucked with a rubber-bulbed pipette, and this was spread upon four slides for examination. It was found advantageous to use coverslips, so that the high power might be switched on quickly when examining living organisms. During March, April and May a total of 197 specimens were examined. Ciliated protozoa of many genera were abundant and a large number of species of nematodes were observed while it was with human satisfaction I discovered a hookworm egg containing a fully formed and living embryo in the very first slide of the series. It is exceedingly difficult to distinguish positively between a motile newly hatched hookworm larva and a parasitic strongyloid, while some of the young larva found seemed to have some characteristics of both; but this difficulty of identification does not obtain in the case of the ovum and of the mature (encapsulated) hookworm larva, and it was possible to say definitely that of the fifty-six tanks examined, the effluents from no less than nineteen contained eggs or larvae capable of transmitting hookworm disease, while fifteen contained roundworm eggs, and one showed tape worm eggs.

Usually, but not always, the eggs were found in the unfiltered effluent and the larvae in the filtered; but in at least one case (Chive Jute Mills Tank 2) an undoubted ripe hookworm larva was found in the unfiltered specimen, indicating that development had probably occurred in the tank itself.

The most heavily infected specimens came from tanks which were overworked and sludged up; but this condition is not necessary, for normally worked and underworked tanks also gave infected effluents. It must be remembered, too, that the volume of the samples was at the most twenty ounces in any one case as against thousands of gallons of effluent daily from each tank, so probably some infections have escaped notice.

I have tried the effect of chlorination and find that a small trace of free chlorine, sufficient to show that *B. Coli* have been destroyed, has no perceptible effect on either eggs or larvæ. The questions of the possibility of designing a septic tank which will be an effective trap for hookworm ova, and of finding a practical means for removing larvæ from the effluents of existing tanks, are at present under consideration and I hope in due course to submit a further note on the matter.

In the meantime, and until some method has been found for freeing the effluent from ova and larvæ, it seems clear that the discharge from septic tank latrines in hookworm countries should not be used directly as manure.

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[An appendix, giving the list of the actual tanks examined and results, has not been published, but those interested may obtain the list by applying to the Author at the School of Tropical Medicine and Hygiene, Calcutta.—*Editor*.]

## A NOTE ON SOME CULTURAL PHASES OF LEISHMANIA DONOVANI.

BY

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[Received for publication, August 21, 1921.]

On July 16, 1920, one of the kala-azar patients at the Institute died. A small monkey (*M. rhesus*) was taken and its peripheral blood examined. No parasites were found. The patient's spleen was removed within 20 minutes of death, and films from the spleen showed a very heavy infection with kala-azar,—small, torpedo-like forms of L.D. predominating.

A small portion of this heavily infected spleen was macerated in sterile normal saline and 10 c.c. of the emulsion given intra-peritoneally to the monkey with a Roux syringe.

From July 16th to August 11th, films of the monkey's peripheral blood were negative and showed no changes. On October 9th, however, it was found that the whole blood picture had changed (incubation period 85 days). Films showed numerous normoblasts, megaloblasts, polychromatophile erythrocytes, a diminution in the polymorphonuclear leucocyte count and a marked increase in the number of large hyaline mononuclears. It was further found that the spleen had become palpable; and spleen puncture was carried out forthwith. Films of spleen juice were negative; and the N.N.N. cultures became contaminated.

On October 12th (88th day), liver puncture was carried out. Films negative. No cultures taken as media not available. By October 15th (91st day) the monkey was definitely ill. Spleen puncture was again carried out. Films negative. Cultures positive: (*vide later*).

On October 16th adrenalin was given hypodermically and six films taken half an hour later from the peripheral blood and most thoroughly searched. No parasites were found.

On October 18th (94th day), a third spleen puncture was done. The films showed one very doubtful form. The cultures were positive : (*vide later*).

On November 3rd (110th day), a fourth spleen puncture was done. Films were again negative : cultures again positive. An entomological assistant now collected all ecto-parasites from the infected monkey. In doing so, most unfortunately he used chloroform as anaesthetic, and the monkey died under the anaesthetic. An immediate post-mortem examination was carried out. The spleen was found to be three or four times its usual size. The liver was enlarged : but other organs appeared healthy. Films were taken from all the internal organs, including liver, spleen, lungs, kidneys and bone marrow. No parasites could be found in the films. Cultures could not be taken as N.N.N. media was not available at the moment.

It will thus be seen that every attempt to obtain a positive diagnosis by examination of films had been negative ; but that on three occasions N.N.N. cultures gave a rich yield of L.D. flagellates. This finding raises again the important question as to whether there may not exist forms of *Leishmania donovani* in the tissues of infected vertebrate hosts which have not yet been identified, but which alone may be capable of carrying on the extra-human cycle of development.

The N.N.N. cultures of October 15th (91st day), taken when the monkey was definitely ill with kala-azar, showed only the usual type of L.D. flagellates up till October 31st (16th day). At 8-30 p.m. on this date it was noticed that these cultures were dry and further films were taken. The appearances seen in fig. 2 of the Plate LXXII were found. On November 9th (25th day), the appearances seen in fig. 3 of the Plate LXXII were found. Until December 5th, these cultures continued to show both normal flagellates (as in fig. 1) and the granule forms (as in fig. 3).

Now the forms in fig. 2 recall Dr. Row's 'post-flagellate forms' of *L. donovani*, but are perhaps better termed 'aflagellate forms'; whilst those in fig. 3, which are best termed 'granule forms,' are like the 'super-post-flagellate forms' described by him as being the phase of flagellate L.D. most infective to experimental animals. In fresh preparations from these cultures the normal flagellates present appear to be

EXPLANATION OF PLATE LXXII.

Fig. 1. Normal flagellates.

" 2. 'Aflagellate' form.

" 3. Granule forms.

(Figs. 2 and 3 appear to be degenerating phases.)

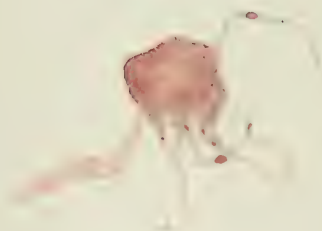
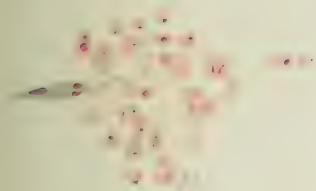
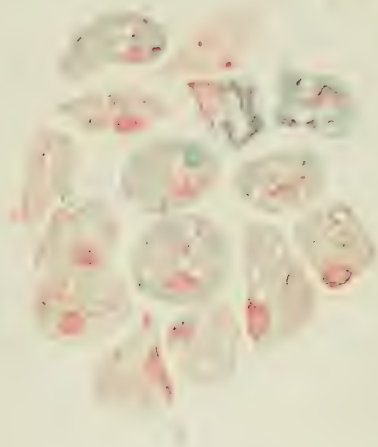
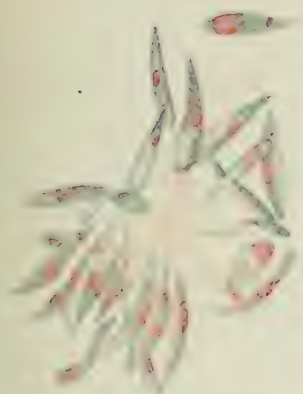
" 4. / Cystic forms from 15 days old culture.

" 5. \

" 6. 'Second generation' flagellates from the same tube seen on  
the 16th day.

## EXPLANATION OF PLATE LXXII.

- the 16th day.
- Fig. 1. Normal flagellates.  
2. Affagellate, form.  
3. Granule forms.  
(Figs. 2 and 3 appear to be degenerating phases)  
4. Cystic forms from 15 days old culture.  
5. Second generation, flagellates from the same tube seen on the 16th day.





actively extruding these granules (this was pointed out to me by Mrs. H. Adie) and the condition of the cultures up to the 51st day is that of a mixture of normal flagellates and granule forms. Yet the cytological picture shown in figs. 2 and 3 is that of degeneration only, with chromolysis of the macronuclei, plasmolysis of the cytoplasm, marked vacuolation, and breaking up of the micronuclei into tiny fragments.—from which the granule forms appear to be derived. It is difficult to form any opinion from one set of such cultures: yet the process in these tubes appears to be one simply of slow degeneration and death of the parasites.

The N.N.N. cultures of October 18th, taken on the 94th day when the monkey was definitely ill clinically, gave a different phase of development. Until the 14th day these cultures showed only the usual and active type of flagellate (as in fig. 1 of the Plate LXXII). The next day, however (November 2nd), the cultures were found to be full of the cystic forms shown in figs. 4 and 5. In fresh preparations these cysts are seen to be spherical bodies of about  $12\mu$  in diameter. Inside the cyst is packed a mass of tiny and very active 'second generation' flagellates. These tiny flagellates are not arranged as in the usual L.D. rosette with their flagella centripetally arranged: but with the flagella centrifugally arranged within the cyst. Active motility is seen to be going on within the cyst: from time to time numerous flagellates are seen to protrude from the cyst: small protuberances arise which are probably due to tiny flagellates trying to escape: and finally, as observed several times in fresh preparations, the cysts rupture and set free swarms of very active and minute flagellates: forms which to a certain extent recall the spirochæte-like forms described by Sir William Leishman. Finally the second generation flagellates are so different from the usual first generation type of flagellate that one can ultimately recognise each variety in films which contain both. The second generation flagellate is very minute, and some of the forms are barely  $5$  to  $6\mu$  in length. In shape they almost resemble protozoal sporozoites. They stain exceptionally well and with very deeply staining macro and micro-nuclei and clear pale blue protoplasm. These forms are shown in fig. 6 and should be compared with the usual type of L.D. flagellate as shown in fig. 1.

The cysts were seen in this set of cultures on November 2nd, the 15th day of culture. Next day there were very few cysts left in the cultures: which now swarmed with the tiny second generation flagellates. Granule forms, as in fig. 3, were found on December 5th,—but are again

probably only degeneration types. On December 5th the culture tubes now show a mixture of both generations of flagellates and granule forms.

The cultures of November 3rd showed only the usual type of L.D. flagellates. Sub-cultures from the cultures of all three dates also have so far only shown the usual type of flagellates.

Now Colonel Cornwall in his very important work on the bed bug has described the transformation of the usual type of L.D. flagellate into first a 'thick tail' phase: and later encystation, with final rupture of the cyst and setting free of very small and very active second generation flagellates. The cultures of October 18th did not show 'thick tails' at any period of observation: and the cyst phase is a very rapid one, barely lasting over 24 hours. It is again difficult to draw conclusions from one set of experiments but the process illustrated in figs. 4, 5, and 6 appears to be one of vital activity rather than of degeneration. Figs. 4 and 5 are bad representations of the process of encystment, as the figures are drawn from Leishman fixed and stained preparations and shrinkage has occurred.

The important point with regard to these cultures, however, is this. There is steadily accumulating evidence that the L. D. body is capable of encystment. In the mid gut of an infected *Cimex rotundatus* cyst formation occurs. It can also occur in N.N.N. cultures. When the extra human cycle of the L.D. body is finally discovered it is likely to be a relatively simple one if the parasite is a true herpetomonad. Whether such encystment, however, is part of the true extra-human cycle, or whether it is a protective reaction on the part of the parasite to unfavourable environment is open to question.

To sum up the history of the infected monkey raises again the question as to whether unrecognised forms of *Leishmania donovani* may not exist in the tissues of its vertebrate host: the aflagellate and granule phases seem to be merely degenerative forms: whilst encystment of the parasite has now been observed in culture, and such encystment may either be a phase of the extra-human cycle or a reaction to unfavourable environment.

I have to acknowledge my indebtedness to Major R. Knowles, I.M.S. for kindly preparing the plate and for the deductions drawn from the cultures. Also I am grateful to Mrs. H. A. Adie and E. C. R. Fox, Esq., Officiating Director, for their kind help and advice.

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REVIEW OF THE POSITION OF THE GENUS  
HÆMOCYSTIDIUM (CASTELLANI AND  
WILLEY, 1904), WITH A DESCRIPTION  
OF TWO NEW SPECIES.

BY

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[Received for publication, July 2, 1921.]

THE first pigmented blood parasite of cold-blooded vertebrates to be described was one discovered by Simond(1) in 1901 in the blood of the Indian river tortoise *Trionyx indicus* (*Chitra indica*) and called by him *Hæmamœba metchnikovi*. In the younger forms of this parasite he described gentle amœboid movements and found that the parasitised red cell hardly suffered at all and that its nucleus usually kept its normal position, colour reaction to stains, and volume. After study of all the forms found by him, he came to the conclusion couched in the following terms: 'L'analogie des deux formes pigmentées de *H. metchnikovi* avec les stades pigmentées des *Hæmamœba* des oiseaux est complète.'

In 1904 Castellani and Willey(2) described a new pigmented blood parasite in the tree gecko *Hemidactylus leschenaultii* and created for it a new genus *Hæmocystidium*, in which they also placed the parasite of Simond. They give no very clear definition of the characteristics of the new genus but appear to attach importance to the following points:—

- (a) Rounded bladder-like form of the parasite.
- (b) Displacement of the host-cell nucleus.
- (c) Presence of pigment.

It will be seen later that (a) and (b) are not necessary characteristics of the genus.

Laveran(3) in 1905 was the next to describe a pigmented blood parasite, of reptiles which he obtained in *Testudo pardalis*. In this species he describes small forms, situated usually near the poles of the infected host-cells, and large forms, lying around the sides or ends of the host-cell nuclei. The parasitised cell is described as little altered although it may be slightly enlarged in the later stages, while the nucleus which may be of normal volume or slightly enlarged, rests usually in its central position or is slightly displaced. He goes on to say: 'L'analogie avec *Haemamoeba danilewskyi* des oiseaux est certaine, comme le fait remarquer Simond, pour le parasite de *Tr. indicus*: l'existence de formes flagellées reste à démontrer pour que le classement de ces parasites dans le groupe des *Haemamoeba* soit indiscutable.'

Sambon(4) in 1907 divided the pigment-producing parasites of reptilia into two genera, basing his classification on the effect produced by the parasites on the parasitised cells.

1. *Plasmodium*, in which he placed *Haemocystidium Simondi*, *Castellani* and *Willey*.

2. *Haemoproteus*, in which he placed *Haemamoeba metchnikovi* *Simond*, and *Haemamoeba testudinis*, *Laveran*.

Wenyon(5) in 1908 described three pigmented blood parasites of reptiles. One of these, which he called *Plasmodium mabuia*, is evidently related to the plasmodia of birds and need not be considered further. Of the other two, he places one, *Haemoproteus agamæ*, from *Agama colonorum*, definitely in the genus *Haemoproteus* on account of the slight action of the parasite on the host-cell and its nucleus and on account of the fact that he was successful in producing exflagellation of the male gametocytes in freshly drawn blood. The other, *Haemocystidium naja* from *Naja haje* and *Naja nigricollis*, he places in the genus *Haemocystidium*, apparently on account of the fact that the gametocytes differed in size and shape from those of *Halteridium* (*Haemoproteus*), the infected cells were enlarged and their nuclei displaced, and there was no evidence of microgamete formation in drawn blood.

That he had some hesitation in placing the parasite in this genus seems apparent from the following quotation from his paper: 'The gametocytes differ in size and shape from those of *Halteridium* but that is insufficient ground on which to base a new genus. The fact that the parasite occurs in a cold blooded host instead of a warm blooded one is also a non-generic character.'

## 816 *Review of the Position of the Genus Hæmocystidium.*

In 1909, Aragao and Neiva(6) described two pigmented parasites of reptiles. One of these appears undoubtedly to have belonged to the genus *Plasmodium*. The other, which they called *Plasmodium diploglossi*, is somewhat difficult to place in its proper category from the description and illustrations of the authors, but the impression one gathers is that they may have been dealing with two different parasites, one a true *Plasmodium* with schizogony in the circulating blood, the other a *Hæmocystidium* present in the form of gametocytes only.

In the same year 1909, Johnston and Cleland (7) described a sixth species from an Australian tortoise, *Chelodina longicollis*.

This species, while like those previously described in general characters, was found to cause no displacement of the nucleus nor distortion of the host-cell. Referring to the formation of a new genus by Castellani and Willey they say: 'But in the above somewhat vague definition of the genus displacement of the nucleus assumes an important position, though, in spite of the absence of this characteristic in Simond's parasite, the latter is included by Castellani and Willey in their genus. It must therefore follow that, if the genus is to hold good, displacement of the nucleus is not a necessary character. We accordingly suggest that the genus be amended as follows: In early stages the parasite is irregular or slightly amœboid; in later stages oval (? schizonts) or rounded, turgid, more or less bladder-like (? gametocytes); it produces melanin granules, vacuoles are frequently present, and there may or may not be displacement of the nucleus of the red cells; and the host is reptilian.'

They go on to say that 'it can be at once distinguished from *Halteridium* by the absence or at least great rarity of halter-like forms, and by displacing the nucleus in one species at least.' This last statement must in the writer's opinion be regarded as at least open to question, since 'halter' forms are by no means rare and, indeed, in some species are the prevailing form, so much so that slides from the peripheral blood are almost indistinguishable from those of birds with typical 'halteridium' parasites. In any case, if the genus as described by Johnston and Cleland is allowed to contain both species displacing and those not displacing host-cell nuclei, it is no greater strain on the elasticity of generic characters for it to contain both species producing chiefly 'halteridium' like forms and those only rarely producing such forms.

The seventh and eighth species of the genus under consideration were described by Bouet (8) in 1909. One of these, *Plasmodium mesali*, was found in a snake not definitely identified from Haute-Côte d'Ivoire and Haute-Senegal, Niger. Here, as in species previously described, both young and mature forms were encountered. The latter are described as halteridial forms encircling the host-cell nucleus, but frequently displacing the latter and deforming the contour of the parasitised cell which is also slightly enlarged. The other form, *Plasmodium roumei*, was obtained in a land tortoise, *Cinixys belliana*, from the Haute-Côte d'Ivoire and from Dahomey. Here again the shape of the parasite was of the halteridium type. The nuclei of parasitised cells however were peripherally displaced, and the cells were somewhat enlarged but without deformation of the contour. Bouet considered that the blood forms approached more nearly to *Haemoproteus* than to *Plasmodium* but that until more of the life cycle was known they should be placed in the genus *Plasmodium* (*Haemamoeba*). The reasoning does not seem quite clear since it would seem more logical, in the absence of knowledge of the life cycle, to place the parasite in the genus towards which its known characters make the nearest approach.

In 1910, Dobell (13) made a further study of the parasite of Castellani and Willey and described schizogony in the circulating blood. According to him 'schizogony occurs in the red corpuscles of the circulating blood of the gecko. It is remarkable in that it consists usually in simple bipartition thereby differing markedly from the schizogony of the malaria parasites and other pigmented intra-corporcular forms. Rarely four merozoites are found instead of two.' The objections one would raise to this interpretation of the forms alluded to by Dobell are two in number:

(1) The forms described and figured by him might be multiple infections of the red cells with two or more parasites such as commonly occur in heavily parasitised hosts. The division of the chromatin into two or four masses as described may be seen even in parasites which are undoubtedly male gametocytes. (Plate LXXIII, Fig. 17.)

(2) If only simple bipartition, or even division into four merozoites of the adult schizonts occurs, how is one to explain the presence of extremely minute forms.

Franca (9) in 1912, describes a species of *Haemacystidium* from *Cinixys belliana*, Gray, which he identifies with *Plasmodium roumei* Bouet. There is no very evident enlargement of the parasitised cells

but the nuclei are displaced even in young forms. In the same year, 1912, Carini and Rudolf(10) described from a Brazilian lizard, *Mabuia agilis*, a pigmented blood parasite which they called *Plasmodium minasense*. They describe very rare schizogony forms in the circulating blood but one is unable with certainty from their somewhat meagre description to place their parasite in its correct category, although probably it should be placed among the *Plasmodia*.

Also in 1912, Pittaluga(14) described a parasite of *Clemmys africana* which he placed in the genus *Hæmoproteus* and called *H. cajali*. He figures distinctly amœboid forms in various stages of development which closely resemble some of the forms to be presently described in another reptile.

In 1916, Froilano de Mello(11) describes a new species *Hæmocystidium kopki* from a gecko *Hemidactylus brookei*, Gray. In this species, according to the author, the parasitised cell is hypertrophied and there is a loss of hæmoglobin, while the nucleus is displaced to one extremity of the cell, unless the parasite has the form of a halter when the nucleus is not displaced. He describes a complete disappearance of the nucleus by a process of karyolysis, but this statement would seem to need confirmation and no such forms are figured. He describes the formation of microgametes in drawn blood, and comes to the conclusion that the parasite cannot be distinguished from those of the genus *Hæmoproteus* in birds.

Franca(12) in 1917 brought out a new classification of the *Hæmoporida*, in which he classes *Hæmoproteus* and *Hæmocystidium* as two distinct genera in the family *Hæmamœbidæ*, Ross, 1899. The generic characters for these two genera are given by him as follows :—

' *Hæmoproteus*, Kruse, 1890. Synonym *Halteridium*, Labbé, 1894. *Hæmamœbidæ* pigmentés, améboïdes, en forme d'halter, ne déplaçant pas de noyau du globule qu'ils entourent. Schizogonie dans les organes internes. Exceptionnellement schizogonie dans le sang circulant : deux groupes de mérozoïtes à chaque pôle de l'halter.

*Hæmocystidium*, Castellani and Willey, 1904.

*Hæmamœbidæ* pigmentés qui, dans leur phase adulte, sont ovalaires ou arrondis (jamais améboïdes), à contour parfaitement régulier et à cytoplasme vacuolisé et noyau presque invisible. Dimorphisme sexuel très net. Le noyau du globule est précocement repoussé à l'une des extrémités de celui-ci. Schizogonie dans le sang circulant par division binaire ou quaternaire.'

It should be noted that sexual dimorphism which is given as one of the generic characters of *Hamocystidium* is equally evident in *Hamoproteus*.

After this description of generic characters for the genus *Hamocystidium* he gives a list of three species in none of which are all the generic characters present, so far as our present knowledge of them goes. The three species given as examples are :

(a) *Hamocystidium Simondi*, Castellani and Willey, 1904. This corresponds fairly closely to the generic characters except that the male gametocytes are stated by Castellani and Willey to have no vacuoles, and no form of schizogony in the circulating blood is described.

(b) *Hamocystidium roumei*, Bouet, 1909. This parasite is described by Bouet as halteridium-like in form and no schizogony in the circulating blood is described.

(c) *Hamocystidium kopki*, Froilano de Mello, 1916. No schizogony in the circulating blood is described.

In 1918-1919, Aragao(15) brought forward a new classification of the Hemosporidia. He places *Hamoproteus* and *Hamocystidium* in separate families of the super-family Plasmodioidea created by himself, as follows :—

	Family	
	<i>Hamoproteidae</i> ,	Genus <i>Hamoproteus</i> , Kuse 1890.
	Samson, 1906.	
Super-family		
Plasmodioidea.		Genus <i>Plasmodium</i> , Marchalava and Gohl
Aragao, 1919.	Family	1885.
	<i>Plasmodiæ</i> ,	Genus <i>Hamocystidium</i> , Castellani and
	Mesnil, 1903.	Willey, 1904.
		Sub-genus <i>Lactania</i> , Grassi and Falcit.
		1890.

If one now considers the generic characters of *Hamoproteus* one will see that most of them are exemplified in one or other of the described species of *Hamocystidium*. The comparison is best made in the form of a table as follows :—

<i>Hamoproteus</i> .	<i>Hamocystidium</i> .
1. Sexual dimorphism ..	.. Sexual dimorphism in all species.
2. Production of pigment..	.. Production of pigment in all species.
3. Younger forms amoeboid	.. Younger forms amoeboid in <i>H. hamocystidis</i> , <i>H. chelodina</i> and <i>H. grahami</i> described in this paper.

## 820 *Review of the Position of the Genus Hæmocystidium.*

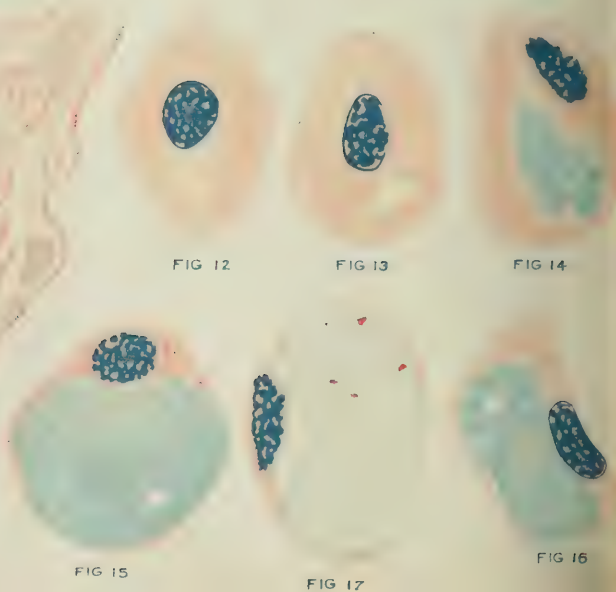
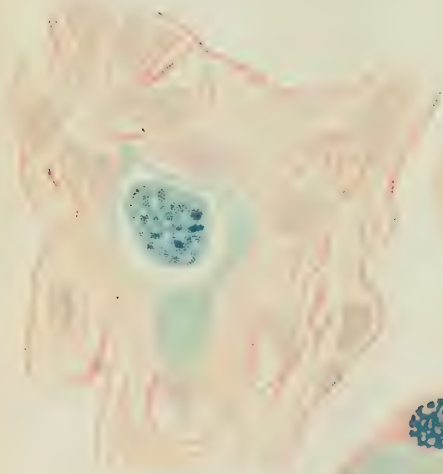
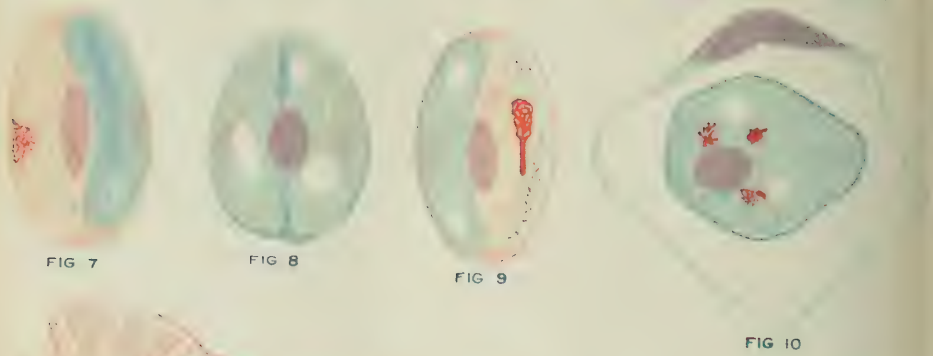
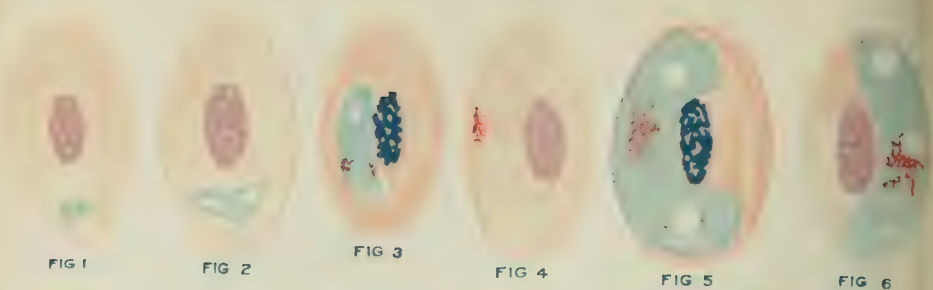
<i>Hæmoproteus.</i>	<i>Hæmocystidium.</i>
4. Nucleus of host-cell not displaced as a rule .. ..	Nucleus of host-cell not displaced as a rule in <i>Hæmoproteus</i> ( <i>Hæmocystidium</i> ) <i>Agamæ</i> , <i>H. chelodinæ</i> , <i>H. testudinis</i> , <i>H. metchnikovi</i> , and <i>H. phyllodactyli</i> (described in this paper).
5. Schizogony in the internal organs (lungs) .. ..	Not yet described.
6. Formation of microgametes in drawn blood .. ..	Formation of microgametes in drawn blood in <i>H. agamæ</i> and <i>H. kopki</i> .

The conclusion one comes to then after a consideration of all the described species of the genus *Hæmocystidium* is that this genus is synonymous with *Hæmoproteus*, since in no case are the characters of the parasite sufficiently distinctive to justify the formation of a new genus and it is therefore considered that the genus *Hæmocystidium*, Castellani and Willey, 1904, should sink before the genus *Hæmoproteus*, Kruse, 1890, which has priority. The distinction, which was made because all the members placed in Castellani and Willey's genus were found in cold-blooded vertebrates, is artificial and as Wenyon has pointed out of no generic significance. The full list of species described from cold-blooded vertebrates to date, including the two new species to be presently described is given below in chronological sequence with the names of the corresponding hosts.

<i>Species.</i>	<i>Host.</i>
<i>Hæmoproteus metchnikovi</i> , Simond, 1901 ..	<i>Trionyx indicus</i> .
" <i>Simondi</i> , Castellani and Willey, 1904 ..	<i>Hemidactylus leschenaultii</i> .
" <i>testudinis</i> , Laveran, 1905 ..	<i>Testudo pardalis</i> .
" <i>agamæ</i> , Wenyon, 1908 ..	<i>Agama colonorum</i> .
" <i>najæ</i> , Wenyon, 1908 ..	<i>Naja hajæ</i> and <i>Naja nigricollis</i> .
" <i>chelodinæ</i> , Johnston and Cleland, 1909 ..	<i>Chelodina longicollis</i> .
" <i>mesnili</i> , Bouet, 1909 ..	<i>Naja</i> sp.
" <i>roumei</i> , Bouet, 1909 ..	<i>Cinixys belliana</i> .
" <i>cajali</i> , Pittaluga, 1912 ..	<i>Clemmys africana</i> .
" <i>kopki</i> , Froilano de Mello, 1916 ..	<i>Hemidactylus brookei</i> .
" <i>phyllodactyli</i> , Shortt, 1921 ..	<i>Phyllodactylus elisæ</i> .
" <i>grahami</i> , Shortt, 1921 ..	<i>Agama nupta</i> , var. <i>fusca</i> .

Doubtful species also are *Plasmodium diploglossi*, Aragao and Neiva, 1909, and *Plasmodium minasense*, Carini and Rudolf, 1912.





## HEMOPROTEUS PHYLLODACTYLII, n. sp.

This parasite was found in an extremely rare species of gecko, *Phyllodactylus elise* of which only one specimen previously existed in the British Museum. It was obtained by the writer at Quritá, on the Persian Frontier, in May 1920.

The description of this species of *Hemoproteus* is limited, with the exception of one form to be presently described, to stages seen in the peripheral circulation. These may be divided into :

- (a) Young forms.
- (b) Intermediate forms.
- (c) Mature forms.

*Young forms.*—Young forms are generally situated at the ends of the erythrocytes and are often pyriform in shape. The smallest forms seen measure 2·3 microns by 2 microns. Stained by Romanowsky stain the protoplasm is of a faint clear blue or violet pink, and there is often a small vacuole situated near one end of the parasite or the vacuole may be absent. In most cases evident chromatin staining is absent. The smallest forms seen contained no pigment. As these small forms grow, pigment grains appear and gradually increase in amount being scattered irregularly through the body, and a second vacuole often makes its appearance. (Plate LXXIII, Figs. 1 and 2.)

*Intermediate forms.*—These are somewhat banana-shaped, with bluntly pointed ends and occupy a position parallel to the long axis of the host-cell, the concavity of the parasite facing the convexity of the host-cell nucleus. The protoplasm of these forms stains in either of two shades of blue, light or dark and they are evidently merely stages in the formation of the mature forms. The pigment is usually aggregated in clumps, often round the periphery of vacuoles in the protoplasm. The chromatin may be in a few distinct aggregations. (Plate LXXIII, Fig. 3.)

*Mature forms.*—These, from their characters as described below, must be looked upon as mature gametocytes. Two forms are present, male and female gametocytes, characterised by different reactions to Romanowsky stains.

(1) *Male gametocytes.* These are stout sausage-shaped forms lying in the position described for intermediate forms. The protoplasm stains a very faint pink or violet pink colour showing faint reticulation. There is a concentration of chromatin at a point near the centre of the parasite, but the chromatin mass may be peripherally placed near the

convex border of the parasite. Generally two or more vacuoles are present, often situated near the extremities of the body. The pigment, of yellow or golden-brown colour, is usually concentrated in larger or smaller masses, the grains of which vary in size or grouped in ring form around the peripheries of the vacuoles. These forms do not cause much displacement of the host-cell nucleus, nor is the host-cell otherwise affected. They measure 13·8 microns by 6 microns (Plate LXXIII, Fig. 4), but as the largest forms curl round the ends of the nucleus, the actual measurements of the parasite if unfolded may be as great as 15·3 microns by 5·2 microns. (Plate LXXIII, Fig. 5 female form.)

(2) Female gametocytes. These in form and size and in their effect on the host-cell resemble the male forms, the only difference being in the staining reaction. The protoplasm of these forms stains a somewhat dense slaty blue with faint reticulation. The vacuoles and the grouping of the pigment are as in the male forms. (Plate LXXIII, Figs. 5 and 6.) The infection in the case of these lizards was often an extremely heavy one but seemed to have no detrimental effect on the host. Besides those already described the following forms were encountered in the peripheral blood:—

- (a) Free forms of male and female gametocytes were not uncommon but were probably the result of trauma in making the slides.
- (b) Double infection of one host-cell with two female gametocytes. (Plate LXXIII, Fig. 8.)
- (c) Double infection of one host-cell with two male gametocytes.
- (d) Double infection of one host-cell with male and female gametocytes. (Plate LXXIII, Fig. 7.)
- (e) Double infection of one host-cell with *Hæmoproteus* and *Hæmogregarine*. (Plate LXXIII, Fig. 9.)
- (f) A doubly infected host-cell phagocytised by a mononuclear leucocyte (? endothelial cell), whose nucleus was pushed to the periphery and flattened out. (Plate LXXIII, Fig. 10.)

In these instances of double infection of one host-cell the apposition of the two parasites to one another when both had reached maturity was so intimate that the actual dividing line could only with difficulty be made out (Plate LXXIII, Figs. 8 and 10), even when one invader was a male and the other a female gametocyte.

In some instances it was found that a young form of *Hæmogregarine* had invaded a host-cell already fully occupied by a double infection with

*Hamoproteus*. From this it would appear that the *Hamogregarine* derives no nourishment at the expense of the haemoglobin of the host-cell, a consideration which is supported by the fact that it produces no pigment, and the inference is that all the *Hamogregarine* requires in this stage of its lifecycle is a 'home' which will act at once as a place of protection and a means of transport during its life in the peripheral circulation.

#### SEARCH FOR SCHIZOGONY IN THE INTERNAL ORGANS.

A prolonged search was made of all the internal organs and the bone marrow by means of smears and sections for stages in schizogony but with the exception of a form in the lung to be presently described nothing was discovered. In the wall of one of the air spaces of the lung an irregularly oblong body surrounded by a cyst-like wall was found. From one side of this cyst-wall a second similar but less densely staining body projected outwards. Both bodies took a deep blue colouration with Giemsa stain and there was an indication of more darkly-staining areas in parts. Both bodies and the cyst-wall contained numerous and uniformly scattered grains of golden-brown pigment. The measurement of the cyst-wall in its greatest diameter was 11.3 microns and of the contained body 7 microns. (Plate LXXIII, Fig. 11.)

I propose to call this species *Hamoproteus phyllodaetyli* n. sp.

#### *HÆMOPROTEUS GRAHAMI* n. sp.

The lizard which is the host of this species is a rock lizard *Agama nupta*, var. *fusca*, common throughout W. and N.W. Persia wherever the conditions are suitable for it.

In the case of this *Hamoproteus* only forms present in the peripheral circulation were encountered. A prolonged search of the internal organs by means of sections revealed no stages in the schizogony of the parasite. One point of interest however in these sections was that the parasites infecting blood cells in the lungs seemed to produce enormous quantities of pigment in abnormally large grains, the pigment frequently almost obliterating the outlines of the infected cell. This I believe not to be caused by actual production of larger quantities of pigment but to be due to the fact that in ordinary blood smears the cellular elements are stretched out and so their superficial area increased, whereas in well-fixed tissue sections they preserve their more compact pattern

forms, so that the same amount of pigment will appear to be relatively greater in amount.

The forms met with in the peripheral blood were :

- (a) Young forms,
- (b) Intermediate forms.
- (c) Mature forms.

*Young forms.*—These were situated at the ends of the host-cells and the most minute forms measured 3 microns by 1.5 microns.

These smallest forms stained a very faint blue with Romanowsky stain and were devoid of vacuoles, but even the smallest forms seen had some fine pigment granules often arranged around the periphery of the parasite. (Plate LXXIII, Fig. 12.) Their shapes varied from rounded oval to long forms. As they increased in size the amount of pigment also increased and the parasites showed considerable amœboid movement as shown in stained preparations. (Plate LXXIII, Fig. 13.) The host-cells were slightly deformed.

*Intermediate forms.*—These, unlike those of the species previously described, generally continued to occupy the end portion of the host-cell and as they grew in size caused displacement of its nucleus and some deformation of its contour. Even those forms which assumed a lateral position in the host-cell caused some deformation of the latter. The protoplasm of parasitised cells was otherwise unaltered.

These intermediate forms showed vacuoles and the same differential staining of the protoplasm and arrangement of the pigment as the species previously described, but differed in showing a very considerable amount of amœboid movement. (Plate LXXIII, Fig. 14.)

*Mature forms.*—These as in the previous description could be differentiated into male and female gametocytes by the differential staining. Vacuoles and pigment were present as in the first-described species but the vacuoles were neither so large nor so numerous. The mature parasites occupied either the ends of the host-cells as more or less rounded forms (Plate LXXIII, Fig. 15) or the sides as very stout oval forms (Plate LXXIII, Figs. 16 and 17). Whatever the situation occupied by the parasite, there was nearly always some deformation of the contour of the host-cell, whose nucleus was often pushed to the periphery. The protoplasm of the host-cell was otherwise unaltered. In both male and female forms areas of diffuse chromatin staining could be made out, but in a few of the male gametocytes the chromatin took the form of distinct discrete dots. (Plate LXXIII, Fig. 17.)

These mature forms measured in their greatest diameter 12 microns to 14.1 microns according to their shape. Double and treble infection of one host-cell were common. In sections of lungs of the host the capillaries were found crowded with infected cells but no stages in schizogony were encountered.

I propose to call this species *Haemoproteus grahami* n. sp.

In conclusion I desire to express my great indebtedness to Professor Stephens of the Liverpool School of Tropical Medicine for allowing me to make use of his laboratories and for his assistance in obtaining for me literature on the subject of this paper. I also wish to acknowledge the kindness of Miss J. Procter of the British Museum in naming for me the two lizards mentioned as harbouring the parasites described, and in obtaining for me additional material from one of the specimens in her possession.

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## NOTES ON TWO HÆMOGREGARINES OF PERSIAN LIZARDS.

BY

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[Received for publication, July 2, 1921.]

THESE hæmogregarines were encountered in two lizards, *Phyllodactylus elisæ*, and *Agama nupta*, var. *fusca*, from Persia, which I have mentioned in another publication<sup>(1)</sup> as harbouring two new species of *Hæmoproteus*.

The forms met with in the peripheral circulation were in no way distinctive from the usual type of hæmogregarine (*sensu stricto*) met with in terrestrial cold-blooded vertebrates, nor were the respective parasites in the two lizards in any way distinguishable from one another as regards the forms seen in the circulating blood. The stages of schizogony in the internal organs however showed a considerable difference in the sizes of comparable forms in the two series and so a short description of each is given.

### HÆMOGREGARINE OF PHYLLODACTYLUS ELISÆ.

Two forms of schizogony were met with:—

- (a) Schizogony in the red cells, with the production of small cysts and small merozoites. This was found to occur in the red cells contained in the capillaries of the lungs, and in the spleen and liver.
- (b) Schizogony in endothelial cells lining the air spaces of the lungs; with the production of larger cysts and larger merozoites. In no other site were these cysts found.

*Schizogony in the red cells.*

In the three organs mentioned numbers of small cysts contained within the red cells were encountered. The word cyst is here used to denote a compact and discrete body clearly marked off from surrounding structures, as an actual cyst-wall was in this type of schizogony very difficult to demonstrate.

The earliest stages met with were small cysts in which the chromatin had already undergone division to produce numerous small, rounded aggregations of chromatin closely packed together and taking a deep bluish-purple colouration with Giemsa's stain. At an early stage the red cell was more or less destroyed and the nucleus apparently extruded so that in most cases the cyst appeared to lie free within the capillary. These cysts measured 6 microns by 5 microns. (Plate LXXIV, Fig. 1.)

Each chromatin mass next attached to itself a small amount of protoplasm so that the cyst became in the next stage an aggregate of minute merozoites (micromerozoites) lying irregularly grouped around a central residual mass. The staining of the chromatin was now much less dense than in the earlier stage. (Plate LXXIV, Fig. 2.)

*Schizogony in endothelial cells of the lung.*

These cysts, as already stated, were only found in endothelial cells lining the air spaces of the lung and sometimes considerable numbers were found in close proximity to one another. The nucleus of the endothelial cell could be seen flattened out at the periphery of the cyst. The cysts measured 15 microns by 12 microns. (Plate LXXIV, Fig. 3.) In contrast to the cysts previously described these contained comparatively large merozoites (macromerozoites) applied to one another like closely-packed sausages. The individual merozoites measured 6·5 microns by 1·8 microns. Each had a clearly-staining nucleus often placed nearer to one extremity. (Plate LXXIV, Fig. 3.)

I suggest for this hæmogregarine the name of *Hæmogregarina procteri* n. sp.

*HÆMOGREGARINE OF AGAMA NUPTA VAR. FUSCA.*

In this form only stages of schizogony comparable to the larger type of cyst in the species already described were met with. No forms of schizogony with the production of the smaller type of cyst were encountered either in red cells or in endothelial cells. In this respect the hæmogregarine differs from one previously described by the writer in an

725-2



Fig. 1



Fig. 2



Fig. 3



Fig. 4



Fig. 5

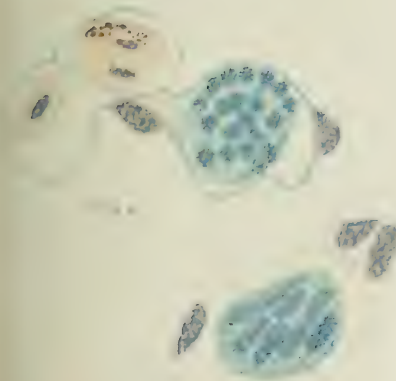


Fig. 6

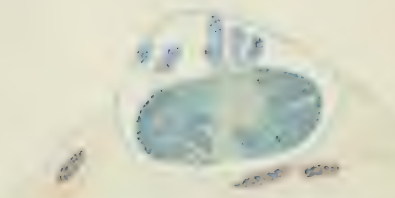


Fig. 7



allied species of *Agama* from the Himalayas<sup>(2)</sup>. The earliest stages of schizogony appeared to be small, oval trophozoites, occasionally seen in small groups or pairs. These apparently entered endothelial cells lining the air spaces of the lungs and sometimes two were encountered in the same cell. (Plate LXXIV, Fig. 4.) They measured 6 microns by 5 microns. The nucleus was dense and the protoplasm of a clear, bluish-violet colour with Giemsa's stain. The trophozoite increased in size and the protoplasm became vacuolated in appearance, while the nucleus remained compact and dense. (Plate LXXIV, Fig. 5.) The trophozoite increased still further in size and the chromatin became divided up into a large number of rounded granular masses. The cytoplasm was of a deep blue and granular in appearance. (Plate LXXIV, Fig. 6.) At a still later stage the chromatin masses became elongated and the granules of chromatin were less densely grouped. (Plate LXXIV, Fig. 7.)

The last stage, or mature cyst, was of large size, measuring 20 microns by 10 microns, while the containing endothelial cell in the cyst figured measured 23·8 microns by 13·2 microns. (Plate LXXIV, Fig. 8.)

The chromatin masses are now seen to be each associated with a small amount of protoplasm, the chromatin or nucleus of the merozoite occupying one end. In the mature cyst figured the merozoites are seen to be already escaping from the cyst which contains a large amount of granular residuum. These merozoites measured 5 microns by 3·3 microns. For this haemogregarine I propose the name of *Haemogregarina percomsi* n. sp.

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## A NEW SERUM TEST FOR KALA-AZAR.

BY

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NOTE ON THE APPLICATION OF THE TEST TO THE DIAGNOSIS OF  
KALA-AZAR BY MAJOR P. S. MILLS, I.M.S.

[Received for publication, November 17, 1921.]

THE technique of the performance of this test<sup>1</sup> is similar to that described by Gaté and Papacosta as the 'Formol-gel test for syphilis'<sup>2</sup> but the results which are obtained if the serum of Kala-azar patients is tested are quite different from anything that is described by these two writers. The specificity of the test is in no way dependent on the gel formation but on the accompanying formation of a white opacity, so that not only is the name 'Formol-gel test for Kala-azar' inappropriate but is likely to be confused with the 'Formol-gel test for syphilis' and consequent misunderstandings are certain to arise. It is therefore suggested that another name be applied to this test. For reasons which follow we have adopted the name 'Aldehyde test.'

The reaction on which the test is dependent was first noted in the following manner. A quantity of serum was required for certain experiments. The serum was obtained from patients under treatment for Kala-azar by withdrawing the blood from the veins after potassium antimonyl tartrate had been injected. The serum was usually preserved by the addition of carbolic acid. On one occasion, however, formalin was used instead of carbolic acid as the latter was not at hand at the time and also because I had always found the former very satisfactory for preserving bacillary emulsions and, if my memory does not betray me,

high-titre sera during the war. Shortly afterwards it was observed that the lower portion of this serum had solidified and was becoming opaque. Some more serum from another Kala-azar patient was taken and formalin added: a marked opacity occurred. It was then found that the addition of dilute carbolic solution intensified the result. Samples of serum from a few patients, including one who had been diagnosed as a case of chronic malaria but who subsequently turned out to be one of Kala-azar, were tested in this way by the addition of a little dilute formalin and carbolic acid: a similar result was obtained in each case. Normal serum remained quite clear after the addition of the same mixture. About this time my attention was called to the paper by Gate and Papacosta, which I had previously overlooked, by Captain W. C. Spackman, I.M.S., who also gave me his experience with the test in a case of Kala-azar. Subsequently a uniform technique was adopted: *i.e.*, one cubic centimetre of serum was separated and one drop of commercial formalin was added. It was found that by this method a more definite result was obtained.

#### *The test.*

About 5 c.c. of blood is withdrawn from the patient's arm and allowed to stand for a sufficient time for the serum to separate. 1 c.c. of clear serum is then placed in a small test tube  $\frac{1}{2}$  inch diameter, to this one drop of 30 per cent\* formaldehyde in the form of commercial formalin is added, the serum immediately well shaken and placed in a test tube rack at laboratory temperature. The results are as follows:—

#### *The serum from an untreated case of Kala-azar.*

The serum will immediately become viscid, within a minute or two will have 'set' so that the tube can be inverted without the serum being spilled, and will begin to become whitish and opalescent. Within from three to twenty minutes, the time varying with different cases, the whole of the serum will have become absolutely solid and opaque like serum coagulated by heat or the 'white' of a hard boiled egg. (Plate LXXV, Fig. IV.) If the serum is hæmoglobin-stained the coagulated serum will have a pink tinge which will turn chocolate brown after 24 hours.

\* *Note.*—The percentage of formaldehyde in commercial formalin is said to be 40 per cent but the samples in our laboratory have been found to be about 30 per cent.

*The serum from cases of phthisis, leprosy, malaria and certain other conditions.*

Usually no change is at first observed but after half an hour, or in some cases less, the serum becomes jellified and after a few hours a certain degree of opacity may be observed but there is no comparison between this and the complete opacity that occurs in the kala-azar cases. (Plate LXXV, Fig. II.)

After 24 hours jellification occurs in the serum from some cases of syphilis. This is the reaction that is described by Gaté and Papacosta.

*The serum of a healthy person or a patient suffering from some disease not included in the above categories.*

The serum will remain quite clear and fluid for a more or less indefinite period. (Plate LXXV, Fig. I.)

*Notes on the reading of results.*

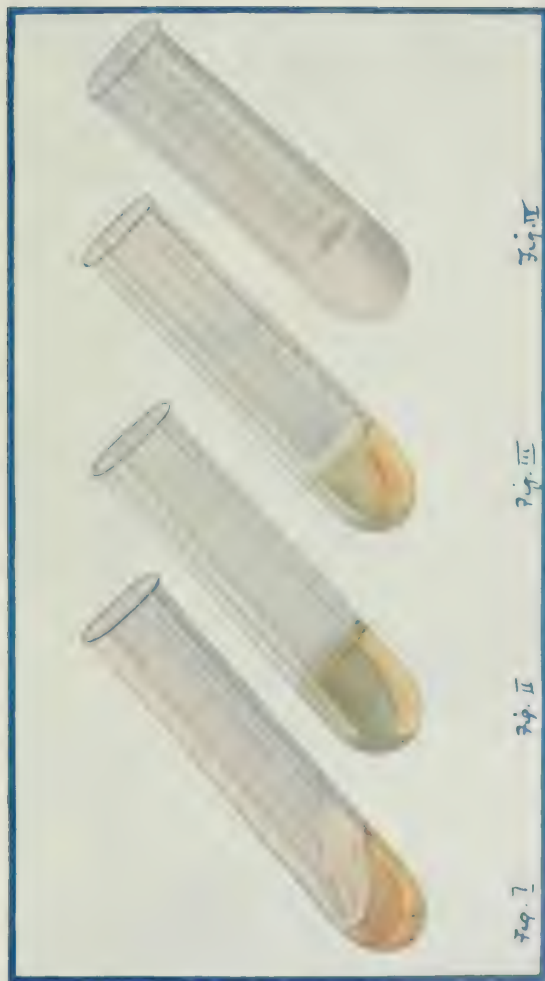
In the case of the serum of very young children with Kala-azar the opacity, after the addition of formalin, may not be absolutely complete, but up to the present we have found that in the case of very young children not suffering from Kala-azar the serum will remain absolutely clear, so that if there is jellification and any degree of opacity in that of a child of, say, three years, the result can safely be considered to be 'positive.'

We have observed recently in two cases of malaria, in which there were a large number of parasites actually present in the blood at the time it was withdrawn, that on addition of the formalin the serum became rapidly solid and assumed quite a marked degree of opacity. (Plate LXXV, Fig. III.) On the first occasion that this occurred we immediately assumed that we were dealing with a case of Kala-azar and were surprised not to find Leishman-Donovan bodies in the spleen, but on the second occasion that we came across a reaction of this kind it was immediately recognised as being similar to the previous one and a diagnosis of malaria was made. This diagnosis was subsequently proved to be correct. Even after standing for 24 hours both these sera were distinctly opalescent and had a characteristic greenish tinge which made them easily distinguishable from the Kala-azar serum which is absolutely opaque and, if previously unstained, a dead white. I was disappointed at getting these two somewhat ambiguous results as they

Fig. I. Normal serum.	Fluid.	—ive.
.. II. Chronic malaria.	Solid but clear.	(—)
.. III. Acute malaria.	Solid and slightly clouded.	(—) (—)
.. IV. Kala-azar.	Solid and absolutely opaque.	+ + +.

- " IV. Kala-ash.  
 " III. Acute malaria.  
 " II. Chronic malaria.  
 Fig. I. Normal serum.

Solid and absolutely opaque.    +++  
 Solid and slightly clouded.    (—) (—) (—)  
 Solid but clear.    (—)  
 Fluid.    — 146.





tend to show that the test is one of degree rather than an absolute test as I had hoped that it would prove to be, and furthermore they make one afraid to trust any modification of this test in which a smaller quantity of serum is used.

In order to measure the opacity we have made use of a small cylindrical cell, 1.25 cm. in diameter and 0.4 cm. deep, the capacity being about 0.5 c.c. Thoma-Zeiss haemocytometer white blood counting pipette also has a capacity of 0.5 c.c. and is therefore a useful mixer. One part of 20 per cent formaldehyde and ten parts of serum are taken into the pipette and rapidly expelled into the cell which is immediately covered with a coverslip. The cell is then placed over some standard print and the time noted at which the print becomes unreadable. In the two malaria cases mentioned above the print was still readable at the end of 24 hours, whereas in all cases of untreated Kala azar it becomes unreadable in from three to twenty minutes or, in extreme cases, two hours. Thus will be a useful method of measuring the patient's progress under treatment, if the disappearance of this reaction is found to be an indication of the improvement of the patient's condition.

These are the only two cases in which any judgment is required in reading the results of the test.

*Signs adopted for recording the results.*

(a) If the serum becomes solid and completely opaque within .. 20 minutes ..	+	+	+
(b) .. .. .. 2 hours ..	+	+	
(c) .. .. .. 24 hours ..	+		
(d) If the serum becomes solid and markedly opalescent but never completely opaque ..	..	(+)	
(e) If the serum becomes solid and slightly opalescent ..	..	(-)	-
(f) If the serum becomes solid but remains quite clear ..	..	(-)	.
(g) If the serum remains fluid and clear for .. 24 hours ..	-	ive	

In the untreated patient (a), (b) and (c) may be considered 'positive,' (d) should be judged according to the age of the patient. And (e), (f) and (g) should be considered as 'negative.'

There is at present at our disposal only one certain method of the diagnosis of Kala-azar, that is, by the recovery of the causative organism

from the patient. The finding of the Leishman-Donovan bodies in the peripheral blood is an excellent method of making a positive diagnosis but the failure to find them does not constitute a negative one. We have found this method of diagnosis a most unsatisfactory one in Calcutta. On only two occasions have we found Leishman-Donovan bodies in the peripheral blood of patients out of over two hundred definite cases examined. It is true that we were not looking for the parasites in most of the cases but either doing blood counts or looking for malarial parasites. Had the Leishman-Donovan bodies been present in any numbers they would most certainly have been observed. On account of the difficulty experienced in keeping culture medium free from contamination during the rains and hot weather we have been compelled to give up this means (*i.e.*, by culture of the peripheral blood) of diagnosis also and to reserve our energies in this direction for checking the results of treatment. We are therefore left with spleen puncture and the microscopic examination of the films for Leishman-Donovan bodies as the only certain means of diagnosis.

It is obvious that before advocating a new method of diagnosis one must check its accuracy by employing it in conjunction with the most certain of previously used methods. We have therefore tried this test in a number of untreated cases in which a spleen puncture has been done.

Table I gives the result of 150 consecutive untreated cases whose condition simulated Kala-azar and on whom a satisfactory spleen puncture had been done. (This table will be found in the Appendix.)

*Analysis of Table I.*

	ALDEHYDE TEST RESULT.		
	Total.	Positive.	Negative.
Cases in which L.-D. bodies were found ..	91	89	2
Cases in which malarial parasites were found ..	22	0	22
Cases of Leukæmia ..	2	0	2
Cases in which no parasites were found ..	35	1	34

It will be seen from the above analysis that the spleen puncture findings and the results of the Aldehyde test correspond in 147 out of 150 cases, that is in 98 per cent of cases.

Case number 38 of this series, the case in which the spleen puncture was negative and the aldehyde test positive, was probably a true case of Kala-azar. She was a woman aged 38; she had had continuous fever for some months; she had a large spleen, a palpable and tender liver, oedema of the feet and she was anæmic. She has now been under treatment by intravenous antimony for two months and is very considerably improved. She has had no fever for over a month; she has no oedema; her spleen and liver are decreased in size and she is less anæmic.

TABLE I.

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test.
1	20-6-21	B. C. S. No. 89	Male	41	L.-D. bodies	Positive.
2	20-6-21	M. No. 93	Male	21	L.-D. bodies found	Positive.
3	20-6-21	R. No. 95	Male	15	No parasites	Negative.
4	20-6-21	H. N. No. 102	Male	19	L.-D. bodies	Positive.
5	20-6-21	A. C. No. 105	Male	30	L.-D. bodies	Positive.
6	28-6-21	N. No. 105	Male	40	No parasites	Negative.
7	1-7-21	Miss E. No. 118	Female	15	L.-D. bodies	+ +
8	2-7-21	J. K. No. 121	Male	40	L.-D. bodies found	Positive.
9	11-7-21	L. B. No. 126	Male	6	L.-D. bodies	+ + +
10	15-7-21	S. S. No. 131	Male	35	L.-D. bodies	( )
11	15-7-21	J. S. No. 133	Male	32	No parasites	( )
12	18-7-21	L. H. No. 136	Male	15	L.-D. bodies	( )
13	18-7-21	R. M. No. 148	Male	36	L.-D. bodies	( )
14	21-7-21	J. N. K. No. 149	Male	7	L.-D. bodies	( )
15	21-7-21	A. No. 157	Male	15	L.-D. bodies	( )
16	21-7-21	M. K. No. 122	Male	21	L.-D. bodies	( )
17	21-7-21	A. No. 147	Male	10	No parasites	( - )
18	22-7-21	A. G. No. 152	Female	12	L.-D. bodies	( )
19	22-7-21	T. H. No. 153	Male	8	L.-D. bodies	+ + +
20	22-7-21	P. S. No. 154	Male	27	No parasites	( - )
21	23-7-21	C. Ward No. 53	Male	51	L.-D. bodies	+ + +

TABLE I.—*contd.*

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test.
22	23-7-21	S. J. No. 155	Female	11	No parasites	— ive.
23	26-7-21	M. No. 145	Female	48	L.-D. bodies	++ +
24	26-7-21	P.B. B. No. 158	Male	19	L.-D. bodies	++ +
25	26-7-21	M. No. 150	Male	40	L.-D. bodies	++ +
26	26-7-21	S. No. 141	Male	15	Malarial parasites, M. T.	(—)
27	26-7-21	R. No. 157	Male	12	Malarial parasites	— ive.
28	26-7-21	P.S. Ward No. 200	Male	16	L.-D. bodies	++ +
29	26-7-21	N. No. 164	Male	32	No parasites	— ive.
30	26-7-21	S. No. 159	Male	32	L.-D. bodies	++ +
31	26-7-21	R. No. 160	Male	12	No parasites	(—)
32	26-7-21	C. No. 162	Female	16	No parasites	(—)
33	26-7-21	S. No. 163	Male	16	L. D. bodies	++ +
34	26-7-21	R. L. No. 161	Male	28	L.-D. bodies	++ +
35	26-7-21	C. No. 135	Male	25	L.-D. bodies	++ +
36	26-7-21	N. N. No. 165	Male	19	Malarial parasites, B. T.	(—)
37	27-7-21	S.M.B. No. 166	Male	29	Malarial parasites, M. T.	(—)
38	27-7-21	M. No. 169	Female	36	No parasites found	++ +
39	27-7-21	D.-J. No. 168	Male	9	L.-D. bodies	++
40	27-7-21	M. K. No. 167	Male	27	L.-D. bodies	++
41	27-7-21	S. No. 138	Male	9	L.-D. bodies	++ +
42	27-7-21	H. No. 156	Male	27	L.-D. bodies	++ +
43	27-7-21	L. R. No. 156	Male	16	L.-D. bodies	++ +
44	27-7-21	U. No. 170	Female	30	Malarial parasites	(—)
45	29-7-21	A. R. N. No. 139	Male	14	L.-D. bodies	++ +
46	29-7-21	M. A. No. 172	Male	18	L.-D. bodies	++ +
47	29-7-21	R. Ward No. 244	Male	50	L.-D. bodies	++ +
48	30-7-21	A. Taylor	Male	5	L. D. bodies	++ +
49	4-8-21	S. No. 175	Male	23	Malarial parasites	(—)
50	4-8-21	K. B. No. 176	Male	40	L.-D. bodies	++ +

TABLE 1.—*contd.*

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test.
51	4-8-21	B. A. No. 177	Male	15	L.-D. bodies	— + +
52	4-8-21	James	Male	35	L.-D. bodies	— + +
53	4-8-21	A. A. Ward No. 237	Male	13	L. D. bodies	— + +
54	5-8-21	M. M. S. Ward No. 236	Male	20	Malarial parasites	— ive.
55	5-8-21	Ward No. 252	Male	40	L. D. bodies	+ + +
56	5-8-21	N. G. No. 179	Male	39	No parasites	( — )
57	5-8-21	T. G. No. 180	Male	14	L.-D. bodies	— + +
58	5-8-21	A. K. No. 181	Male	15	No parasites	— ive.
59	10-8-21	Ward No. 221	Male	30	No parasites	( — )
60	10-8-21	Ward No. 210	Male	20	L.-D. bodies	— + +
61	10-8-21	S. No. 183	Male	20	L. D. bodies	— + +
62	10-8-21	L.M.B. No. 184	Male	14	No parasites	— ive.
63	10-8-21	K.C.B. No. 145	Male	30	L.-D. bodies	+ +
64	12-8-21	Ward No. 220	Male	25	L. D. bodies	+ +
65	12-8-21	Ward No. 217	Male	50	L.-D. bodies	+ + +
66	12-8-21	B. No. 186	Male	6	Malarial parasites	— ive.
67	15-8-21	C. No. 188	Male	35	L.-D. bodies	— + +
68	15-8-21	H. C. C. No. 190	Male	54	L.-D. bodies	— + +
69	16-8-21	Ward No. 195	Male	24	L.-D. bodies	— + +
70	17-8-21	H. No. 191	Male	18	Malarial parasites	( — )
71	17-8-21	I. N. No. 192	Male	20	No parasites	( — )
72	17-8-21	S. No. 194	Male	35	No parasites	— ive.
73	17-8-21	Miss S. No. 195	Female	10	L.-D. bodies	+ + +
74	17-8-21	Miss N. No. 196	Female	17	L.-D. bodies	+ + +
75	17-8-21	M. L. No. 197	Male	17	L.-D. bodies	+ + +
76	17-8-21	S. No. 198	Male	11	L.-D. bodies	+ + +
77	17-8-21	S. No. 199	Male	30	L.-D. bodies	+ + +
78	17-8-21	T. D. No. 124	Female	14	L. D. bodies	+ + +

TABLE 1.—*contd.*

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test.
79	17-8-21	J. No. 200	Female	11	L.-D. bodies	+++
80	17-8-21	R. D. No. 202	Male	20	L.-D. bodies	+++
81	17-8-21	E. B. No. 203	Male	22	L.-D. bodies	+++
82	17-8-21	H. H. No. 204	Male	17	Malarial parasites	(-)
83	22-8-21	M. No. 205	Male	18	L.-D. bodies	+++
84	22-8-21	R. J. No. 207	Male	30	Malarial parasites	(+)
85	23-8-21	P. No. 208	Male	30	Malarial parasites	(-)
86	23-8-21	S. T. No. 209	Male	35	No parasites	(-)
87	24-8-21	Ward No. 217	Male	30	L.-D. bodies	+++
88	24-8-21	H. L. G. No. 211	Male	42	L.-D. bodies	+++
89	24-8-21	T. No. 212	Male	18	L.-D. bodies	+++
90	25-8-21	P. No. 213	Male	25	L.-D. bodies	+++
91	25-8-21	S. G. R. No. 214	Male	23	L.-D. bodies	+++
92	25-8-21	Ward No. 220	Male	32	L.-D. bodies	+++
93	25-8-21	H. B. No. 218	Male	38	L.-D. bodies	+++
94	30-8-21	Ward No. 250	Male	29	No parasites	(-)
95	30-8-21	S. No. 219	Male	26	No parasites	(-)
96	31-8-21	A. No. 220	Male	50	L.-D. bodies	+++
97	31-8-21	B. K. D. No. 221	Male	30	No parasites	(-)
98	31-8-21	P. P. G. No. 4	Male	17	No parasites	(-)
99	31-8-21	B. N. M. No. 222	Male	22	No parasites	(-)
100	31-8-21	P. D. No. 223	Male	26	Malarial parasites	(-)
101	1-9-21	K. No. 224	Male	10	L.-D. bodies	+++
102	1-9-21	A. H. No. 226	Male	15	L.-D. bodies	+++
103	1-9-21	Ward No. 54	Male	36	No parasites, leukaemia	(-)
104	2-9-21	S. No. 227	Male	15	No parasites	(-)
105	2-9-21	L. H. No. 228	Male	15	L.-D. bodies	++
106	2-9-21	S. No. 229	Male	15	L.-D. bodies	+++
107	7-9-21	B. No. 230	Male	22	No parasites	(-)

TABLE I.—*contd.*

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test
108	7-9-21	J. G. No. 231	Male	18	L.-D. bodies	—
109	7-9-21	N. No. 232	Male	16	L.-D. bodies	— ive.
110	7-9-21	R. G. No. 233	Male	32	L. D. bodies	—
111	7-9-21	B. No. 235	Male	13	No parasites	— ive.
112	10-9-21	Ward No. 225	Male	17	L.-D. bodies	—
113	10-9-21	H. No. 239	Male	16	Malarial parasites	—
114	10-9-21	H. L. M. No. 240	Male	25	No parasites	— ive.
115	19-9-21	S. No. 245	Male	28	No parasites	— ive.
116	19-9-21	G. M. No. 247	Male	12	L.-D. bodies	—
117	19-9-21	S. A. No. 248	Male	20	L.-D. bodies	—
118	21-9-21	K. C. No. 249	Male	20	L.-D. bodies	—
119	21-9-21	K. M. No. 250	Male	29	L. D. bodies	—
120	21-9-21	H. G. B. No. 251	Male	26	No parasites	— ive.
121	21-9-21	R. No. 252	Male	16	Malarial parasites	—
122	21-9-21	W. I. B. No. 253	Male	27	L.-D. bodies	—
123	21-9-21	N. w. No. 254	Male	9	Malarial parasites, B. T.	—
124	21-9-21	N. No. 256	Male	40	No parasites, leukæmia.	— ive.
125	22-9-21	K. No. 257	Male	30	L.-D. bodies.	—
126	22-9-21	M. No. 258	Male	20	Malarial parasites	( — )
127	26-9-21	Ward No. 215	Male	45	No parasites	( — )
128	26-9-21	Ashu No. 261	Male	23	L. D. bodies	+ + +
129	26-9-21	I. No. 263	Female	16	L.-D. bodies	—
130	26-9-21	N. No. 264	Female	25	L. D. bodies	—
131	26-9-21	G. No. 266	Male	14	L. D. bodies	+ + +
132	28-9-21	H. No. 267	Male	30	Malarial parasites	—
133	28-9-21	A. M. No. 269	Male	11	Malarial parasites, B. T.	—
134	30-9-21	O. M. No. 270	Male	32	L.-D. bodies	+ + +
135	30-9-21	H. No. 271	Male	22	No parasites	( — )
136	30-9-21	T. No. 273	Male	35	No parasites	—

TABLE I.—*concl'd.*

No.	Date.	Patient.	Sex.	Age.	Spleen puncture findings.	Aldehyde test.
137	30-9-21	D. N. No. 274	Male ..	16	L.-D. bodies ..	+ + +
138	3-10-21	A. No. 275	Female ..	16	Malarial parasites, B. T.	( - )
139	3-10-21	D. No. 277	Male ..	10	L.-D. bodies ..	+ + +
140	3-10-21	R. H. No. 278	Male ..	30	No parasites ..	( - )
141	3-10-21	M. No. 279	Male ..	22	L.-D. bodies ..	+ + +
142	5-10-21	M. O. No. 280	Male .	34	No parasites ..	( - )
143	5-10-21	M. No. 282	Male .	30	No parasites ..	( + )
144	6-10-21	G. N. No. 283	Male ..	20	L.-D. bodies ..	+ + +
145	13-10-21	R. F. No. 285	Male .	13	L.-D. bodies ..	+ + +
146	13-10-21	K. No. 286	Male ..	11	L.-D. bodies ..	+ + +
147	13-10-21	A. No. 287	Male ..	30	Malarial parasites ..	- ive.
148	13-10-21	G. K. No. 288	Male ..	32	No parasites ..	( - )
149	13-10-21	A. G. No. 289	Male ..	28	Malarial parasites, B. T.	( - )
150	13-10-21	M. N. No. 291	Male ..	40	No parasites ..	( + )

Numbers 14 and 109, the two cases in which the aldehyde test was negative but in whose spleens the parasites of Leishmaniasis were found, were clinically very much alike. They gave a history of a year and a month, respectively. They denied having lost weight and both looked well nourished. They gave no history of bleeding from the gums or nose: were only slightly if at all anæmic: their pulses were slow and their spleens not markedly enlarged. The clinical diagnosis in each case, prior to spleen puncture, had been chronic malaria. The former patient attended for treatment without much change in his condition for about a month and then discontinued of his own accord. The latter did not attend for treatment.

*The aldehyde test and the Wassermann reaction.*

As the formol-gel test, the father of this test, was put forward as a test for syphilis it was felt that it would be interesting to compare the results of the aldehyde test with those of the Wassermann reaction. The

Wassermann reaction was done in 86 of the 150 cases reported in table I. This work was carried out by the Imperial Serologist. Table II gives these results.

TABLE II.

	Total.	WASSERMANN REACTION.		
		Positive.		Negative.
		Strongly.	Partially.	
Cases in which the aldehyde test was 'positive' .. .. .	60	12	7	41
Cases in which the aldehyde test was 'negative' .. .. .	26	4	5	17

The figures seem to suggest that there is very little, if any, relationship between the two reactions.

#### Further controls.

Although the test has been done on nearly 60 non-kala-azar cases amongst the spleen-puncture series, it was thought advisable to do it on a few more samples from patients suffering from other diseases. Fifty cases were chosen in whom a definite diagnosis of some other disease had been made and their serum was subjected to the aldehyde test. Table III gives these results. The patients were all adults. It was only in the

TABLE III.

*Controls. — Cases with a definite diagnosis of some condition other than Kala-azar.*

No.	Patient.	Diagnosis.	Aldehyde test
1	No. 3 Ward ..	Enteric ..	— ive.
2	No. 45 O. P. ..	Filariasis ..	— ive.
3	Leper 'A' ..	Leprosy, advanced ..	(—) (—)
4	Leper 'B' ..	Leprosy, moderately severe ..	(—)
5	Leper 'C' ..	" ..	(—)
6	Leper 'D' ..	" ..	— ive.
7	Leper 'E' ..	" ..	(—)

TABLE III.—*contd.*

No.	Patient.	Diagnosis.	Aldehyde test.
8	Leper 'F' ..	Leprosy, moderately severe ..	— ive.
9	Leper 'G' ..	" " " ..	(—)
10	Leper 'H' ..	" " " ..	(—)
11	C. F. G. ..	Dysentery ..	— ive.
12	No. 182 O. P. ..	Syphilis (Wassermann positive) ..	— ive.
13	T. B. No. 1 ..	Advanced pulmonary tuberculosis ..	(—)
14	T. B. No. 3 ..	" " " ..	(—) (—)
15	T. B. No. 7 ..	" " " ..	(—)
16	T. B. No. 17 ..	" " " ..	(—)
17	T. B. No. 19 ..	" " " ..	(—)
18	T. B., O. P. a. ..	Pulmonary tuberculosis ..	(—)
19	T. B., O. P. b. ..	" " " ..	— ive.
20	T. B., O. P. c. ..	" " " ..	(—)
21	T. B., O. P. d. ..	" " " ..	(—)
22	T. B., O. P. e. ..	" " " ..	(—) (—)
23	No. 248 Ward ..	Malignant disease ..	(—)
24	No. 206 ..	Pelvic inflammation ..	— ive.
25	Jew ..	Appendicitis ..	— ive.
26	No. 160 ..	Advanced T. B. lungs ..	(+)
27	No. 167 ..	Leukæmia, spleeno-medullary ..	— ive.
28	No. 261 ..	Early tuberculosis, lungs ..	— ive.
29	No. 272 ..	B. T. malaria ..	— ive.
30	No. 232 ..	M. T. malaria ..	(—)
31	Ward No. 228 ..	Gastric ulcer ..	— ive.
32	Ward No. 230 ..	Malaria ..	— ive.
33	Ward No. 232 ..	Morbus Cordis ..	— ive.
34	Ward No. 239 ..	Nephritis ..	— ive.
35	Ward No. 243 ..	" " " ..	— ive.
36	Ward No. 244 ..	Hemiplegia ..	— ive.
37	Ward No. 209 ..	Gonorrhœa ..	(—)
38	Ward No. 214 ..	M. C. Mitral ..	— ive.
39	Ward No. 216 ..	" " " ..	— ive.
40	Ward No. 219 ..	Dysentery ..	(—)
41	Ward No. 221 ..	Morbus Cordis ..	(—)
42	Ward No. 200 ..	Malaria ..	— ive.
43	Ward No. 217 ..	Beri-Beri ..	— ive.
44	Ward No. 206 ..	Malaria ..	— ive.
45	M.'s case ..	Tuberculosis ..	(—)
46	McV.'s case. a. ..	Ankylostomiasis ..	— ive.
47	McV.'s case. b. ..	" " " ..	— ive.
48	No. 198 ..	Broncho-pneumonia ..	— ive.
49	No. 234 ..	Lobar pneumonia ..	— ive.
50	No. 251 ..	Malaria, M. T. ..	(—)

tuberculosis and leprosy cases that any clouding of the serum was observed. It was not considered necessary to do the test with the serum from normal individuals and from cases of uncomplicated syphilis as this has already been done by the originators of the Formol-gel reaction and other observers with apparently uniformly '— ive' or '(—)' results.

*The test in Kala-azar cases under treatment.*<sup>2</sup>

The results which are obtained if the serum of patients under antimony treatment be tested vary between strongly positive and absolutely negative. There is a decided tendency for the reaction to disappear as the treatment progresses but we are not yet in a position to say to what extent we can rely on this test as an indication of the progress of the patient towards recovery. If it is found that the progress of the patient can be measured in this way an accurate method of measuring the opacity of the serum will have to be introduced. Possibly some method on the lines of the one that I have already suggested might be used. Tables IV and V give the results of the test in treated Kala-azar cases.

TABLE IV.

*Cases under treatment.*

No.	Date.	Patient.	Age and sex.	Mgms. of antimony salt given.	Result.
1	15-7-21	No. 59	30 male	2,000	(+)
2	15-7-21	No. 54	12 male	1,500	- ive.
3	21-7-21	No. 109	27 female	160	(-) (-)
4	21-7-21	No. 45	13 male	1,400	(+)
5	21-7-21	No. 123	20 male	160	+
6	22-7-21	No. 225 Ward	28 male	2,000	+
7	22-7-21	No. 226 Ward	18 male	160	+++ no gel
8	22-7-21	No. 70	8 female	1,000	- ive.
9	23-7-21	No. 63	17 male	800	- ive.
10	23-7-21	No. 12	35 male	2,000	+ +
11	23-7-21	No. 211 Ward	11 male	2,200	- ive.
12	23-7-21	No. 223 Ward	12 male	1,800	(+)
13	23-7-21	No. 52	25 male	700	+ +
14	23-7-21	No. 35	26 male	2,000	- ive.
15	23-7-21	No. 99	43 male	600	(+)
16	29-7-21	No. 10 Ward	12 female	500	(+)
17	29-7-21	No. 96	9 male	650	(+)
18	4-8-21	No. 78	21 male	1,000	- +
19	4-8-21	No. 48	11 male	1,500	(+)
20	4-8-21	No. 32	7 female	1,000	- ive.
21	4-8-21	No. 13	5 male	800	(+)
22	4-8-21	No. 28	9 female	1,200	- ive.
23	4-8-21	No. 107 Ward	15 female	1,000	+ +
24	4-8-21	No. 121	32 male	500	+
25	4-8-21	No. 59	30 male	2,500	(-)
26	12-8-21	No. 106	40 male	400	+ + +
27	12-8-21	Ezra	28 male	100	+ +
28	16-8-21	Ranao	14 male	1,000	(+)
29	16-8-21	E. B.	12 male	2,000	- Non.

TABLE V.

*Cases in which the aldehyde test was done prior to treatment and again after treatment was considered complete.*

Patient.	Sex and age	Aldehyde test prior to treatment.	Nature of treatment adopted.		Aldehyde test after treatment.
No. 227 Ward. ..	Male 15 ..	+ + +	Intra-muscular	S.A.T.	(-)
No. 10 Ward ..	Male 5 ..	+ +	do.	do.	(-)
No. 118 ..	Female 15 ..	+ +	Intravenous Pot.	A. T.	(-)
No. 53 Ward ..	Male 41 ..	+ + +	Intra-muscular.	S.A.T.	(+)
No. 126 ..	Male 6 ..	+ + +	do.	do.	- ive.

*Suggested modifications.*

As it stands the test can be performed by any medical practitioner who is capable of withdrawing blood from a patient's veins. The veins of small children are sometimes difficult to puncture and there are still some practitioners who hesitate, or are unable, to perform this simple operation even on an adult. One is therefore anxious to find some method which will give equally accurate results without the necessity of venipuncture. This, I think, will be difficult to find.

If blood is drawn from the finger into a Wright's capsule and allowed to clot the test can be performed with the drop of serum thus obtained in a number of ways.

(a) One part of 15 per cent formaldehyde and ten parts of serum can be drawn into a fine Wright's pipette, mixed and sealed. The opacity can easily be observed by holding it against a black background and the consistency can be noted by breaking the pipette. (b) The serum and formaldehyde can be more accurately measured by means of a hæmocytometer pipette and mixed on a slide where the opacity and the consistency can easily be observed.

(c) A drop of serum can be placed on the inside wall of a test tube, held at an angle so that the drop does not run down, in which a few grains of para-formaldehyde have been placed and the test tube heated gently. Formaldehyde is liberated which will solidify a 'positive' serum in the same way that the solution does. All these modifications

tend to decrease the accuracy of the test. The modification that I have found most useful is the following :

Four drops are taken directly from the patient's finger into a small test tube containing 0.5 c.c. of citrate saline (Sodium chloride 0.85 grammes, sodium 0.5 grammes to 100 c.c. of water) in which there is one drop of formalin. If this is left overnight the red blood cells will settle and the supernatant fluid will be milky in the Kala-azar case, clear in the other.

But even with this modification the difference between a '++' result and a '(+)' result is not sufficiently distinct to make the method really reliable.

#### *Notes on the chemistry of the reaction.*

At first we used commercial formalin, the formaldehyde content of which was 30 per cent, but latterly Dr. Sudhamoy Ghosh has kindly prepared a solution of pure formaldehyde in distilled water which we have found gives much clearer results. A 10 per cent solution was used and a drop was added to 1 c.c. of serum. The question of the optimum proportions is one that will have to be investigated. I have found that one part of formaldehyde, by weight, to 200 parts of serum gives a rapid and complete result. If the amount of formaldehyde be increased any extent the reaction will tend to be less marked and if excess of formaldehyde be added the reaction does not occur.

The reaction seems to be essentially an aldehyde one as the next higher aldehyde, acet-aldehyde, also gives the reaction but less completely and more slowly. Six sera were tested with acet-aldehyde, a 40 per cent solution in distilled water being used.

The results were as follows :—

Serum.			Formaldehyde (usual sign used)	Acet-aldehyde. (Readings after 24 hours.)
a.	..	..	+++	Solid and quite opaque.
b.	..	.	+++	Solid and almost opaque.
c.	..	..	++	Solid and opalescent.
d.	..	..	(-)	Solid but clear.
e.	..	..	(=)	Solid but clear.
f.	..	..	— i.e.	Fluid.

The explanation of the reaction is not obvious and an attempt to seek it would, I fear, carry me into the realms of physical chemistry where I should very soon be completely lost. A few facts however have been noted.

The portion of the serum which is actually responsible for the formation of the specific opacity seems to be grouped amongst the eu-globulins. After 33 per cent saturation with ammonium sulphate and subsequent removal of the ammonium sulphate by dialysation from the filtrate, the latter will not give the reaction, whereas the precipitate dissolved in physiological saline and also freed from ammonium sulphate does give it. Again, after dialysation of the serum against 50 times its volume of distilled water, and consequent precipitation of most of the globulins, the speed of the reaction is very markedly increased, in fact on a drop of formalin being added the serum coagulates almost instantaneously, but if dialysation is continued in running distilled water further precipitation of the globulins occurs and the remaining serum fails to react in the characteristic manner with formaldehyde. The precipitated globulins when dissolved in saline give the reaction.

It seems possible that the reaction is in some way dependent on the hydrogen-ion concentration of the serum. The PH is much increased in the serum of Kala-azar patients. Alteration of the PH in either direction will prevent the aldehyde reaction occurring in a 'positive' serum and very light increase in the PH of some negative sera will, in combination with the addition of formaldehyde, cause coagulation to occur. Further investigation is at present being carried out on this point.

My thanks are due to Captain W. C. Spackman, I.M.S., for first drawing my attention to Gaté and Papacostas' paper and thereby considerably stimulating my interest in the subject.

My thanks are also due to Major R. B. Lloyd, I.M.S., Imperial Serologist, for doing the Wasserman reactions, to Dr. Sudhamoy Ghosh, Professor of Chemistry at the Calcutta School of Tropical Medicine, for preparing and estimating the strength of pure formaldehyde solutions and for his valuable advice, and to my assistant Dr. P. Murugesan.

## NOTE ON THE FORMALIN TEST AS APPLIED TO THE DIAGNOSIS OF KALA-AZAR.

BY

MAJOR P. S. MILLS, I.M.S.

WHEN in Calcutta recently, Dr. Napier demonstrated to me the use of the formalin test in the diagnosis of Kala-azar.

It is the practise in the Chapra Dispensary to examine the splenic blood microscopically of cases in which the splenic enlargement is thought to be due to Kala-azar. To submit a patient to prolonged treatment by intravenous injections of tartar emetic does not appear to be justifiable unless the diagnosis of Kala-azar is certain, and up to now it has been very difficult to differentiate between the splenic enlargements caused by Kala-azar and malaria, respectively.

Eleven cases have been tested by both the formalin test and the microscopic examination of the splenic blood for Leishman-Donovan bodies.

In order to have independent observations, Captain R. P. Ghosh was kind enough to carry out the formalin tests. This was done by withdrawing 5 c.c. of blood from the arm of the patient, and allowing the blood to clot in a large bore test tube. One c.c. of the clear serum was poured out into a small test tube, and one drop of formalin added. In a positive case, a white precipitate was formed in a few seconds in the serum, which solidified within two minutes.

The spleen punctures and microscopic examination were done by myself, so that my findings were always independent of those of Captain Ghosh.

In 5 cases we found that both the formalin test and the finding of L.-D. bodies were positive. In 6 cases both the formalin and the microscopical tests were negative. So far, therefore, in the whole series of eleven cases, we have found the two tests to correspond absolutely. Among the 6 cases in which both tests were negative, we found in one

that the serum coagulated at the end of 30 minutes, with the formation of the typical white precipitate.

A table of the cases is appended.

#### REMARKS BY DR. NAPIER.

THE case in which Major Mills describes the formation of 'a typical white precipitate' after a delay of 30 minutes is in all probability a case similar to the two I described in which solidification and marked clouding occurred without complete opacity. At the time that I demonstrated this test to Major Mills I had not come across one of these confusing results.

*Statement showing the formalin reaction of blood sera of Kala-azar patients in Chapra Hospital from 21-8-21 to 21-9-21.*

Serial No.	Name.	Sex.	Age.	Religion.	Chemical diagnosis.	Spleen puncture finding.	Formalin reaction (1 drop formalin to c.c. serum).	REMARKS.
1	Biswanath ..	Male	12	Hindu	Enlarged spleen	L.-D. bodies found.	Positive	..
2	Ram Subhag Tiwary.	"	30	"	"	No L.-D. bodies found.	Negative	..
3	Nasiban ..	Female	13	Moham-medan.	"	Do.	"	..
4	Lal Behari ..	Male	35	Hindu	"	Do.	"	..
5	B h a g w a t Tiwary.	"	22	"	"	L.-D. bodies found.	Positive	..
6	Ram Brich Tiwary.	"	30	"	"	No L.-D. bodies found	Negative	..
7	Pitamber Gir.	"	25	"	"	Heavy infection of L.-D. bodies.	Positive	..
8	Shamdai ..	Female	12	"	"	No L.-D. bodies found	Negative	..
9	S e o p u j a n Ram.	Male	22	"	"	Heavy infection of L.-D. bodies.	Positive	..
10	Bhola Sah ..	"	35	"	"	No. L.-D. bodies found	Negative	The serum coagulates after 30 minutes with the white precipitate.
11	Majeed ..	"	14	Moham-medan.	"	L.-D. bodies found.	Positive	..

All the Spleen punctures were satisfactory and presented no difficulty.

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# GATÉ-PAPACOSTAS REACTION IN LEPROSY.

BY

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[Received for publication, October 5, 1921.]

GATE and Papacostas, while experimenting with pooled sera in connection with their routine Wassermann work, accidentally discovered a new and simple method for the diagnosis of syphilis. They found that the addition of two drops of commercial formalin to about 1·0 c.c. of clear syphilitic serum in a small test tube, and keeping it for 24 to 36 hours, caused the mixture to become more or less solid and jelly-like; while in the case of a negative reaction the mixture remained perfectly fluid. The test has been fully described by the authors in *Compt. Rend. Soc. Biologie*. Nov. 1920, p. 1432. In their experiments the results of this so-called 'Formol-gel' test and the Wassermann reaction coincided in 85 per cent of cases. No preliminary inactivation of the serum was necessary, nor was the reaction modified by incubation of the mixture.

Comparative experiments were carried out in the Bombay Bacteriological Laboratory with a view to test the value of this reaction. The results were as follows:—

In our first series of experiments, out of 186 serums of various cases, 44 showed a markedly positive (+++) Wassermann reaction, but only 30 out of these, *i.e.*, approximately 68 per cent, gave a positive Formol-gel test.

Out of 15 serums which showed ++ Wassermann reaction, none gave a positive Formol-gel test.

Out of 124 serums which were negative to the Wassermann test, eight reacted positively to the Formol-gel reaction. Of these eight, one was a case of cutaneous Leishmaniasis, another of rectal stricture, and the remaining six were suffering from leprosy.

Three serums proved to be anticomplementary, but they gave a negative Formol-gel reaction.

We were interested in the results obtained in these six cases of leprosy, and consequently decided upon performing this test on an extensive scale among lepers.

A second series of experiments was therefore carried out among the inmates of the Aeworth Leper Asylum at Matunga in Bombay, and 116 serums were examined. The cases of leprosy were not specially selected; nodular anæsthetic and mixed cases were included indiscriminately.

The result of our experiment showed that *every one of these cases of leprosy gave a definitely positive Formol-gel reaction*. Our test was carried out in every instance with inactivated serum only.

From these observations it appears to us that the Gate-Papacostas reaction is likely to prove a valuable serological test in the diagnosis of leprosy.

We take this opportunity of thanking Dr. Rodrigues, Superintendent, Aeworth Leper Asylum, for kindly placing the cases of leprosy at our disposal for carrying out these observations, and Major E. C. Hodgson, D.S.O., I.M.S., the Director of this Laboratory, for his advice.

# AN EXAMINATION INTO THE DEGREE OF EFFICACY OF ANTI-RABIC TREATMENT.

BY

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[Received for publication, December 2, 1921.]

## Part I.

### CONTENTS.

	Pages.
1 Statistical Enquiry .. .. .	852
1·1 Introduction .. .. .	852
1·2 Mortality Rate of Rabies .. .. .	855
1·21 Definition .. .. .	855
1·22 Correction of Death Rates .. .. .	856
1·23 Diminution of Mortality Rates with Continued Existence of Insti- tutes .. .. .	857
1·24 Oriental Population more Exposed to Risk than European .. .. .	857
1·25 Effect of Lateness of Arrival on Mortality Rate .. .. .	858
1·26 Severity of Wounding and Mortality Rates .. .. .	859
1·27 Mortality Rates among Treated and Untreated .. .. .	859
1·28 Constitution of Population and Mortality Rates .. .. .	864
1·29 Incubation Period of Rabies and the Mortality Rate .. .. .	867
1·3 : 1·31 Mortality Rates, Rabies .. .. .	869
1·4 Legal Enactments and Rabies Mortality .. .. .	872
1·5 Conclusions .. .. .	874

## 1 STATISTICAL ENQUIRY.

**1·1 INTRODUCTION.**—An enquiry into the degree of efficacy of a form of treatment which has behind it the authority of one of the greatest names of science and which has been practised for upwards of 35 years would seem to be superfluous. That it is not superfluous it is our purpose

to show; and although we do not maintain that our researches have carried us to finality of conclusion, yet we may assuredly claim that they render essential a thorough re-examination of the whole subject. This is evidently also the feeling of a considerable section of workers on the subject of rabies. Babes proposed that there should be a conference to settle the subject of the best mode of treatment, a settlement which is rendered especially necessary in view of the very great diversity at present prevailing. This proposal took concrete shape in the suggestion, or rather the arrangements, made for a congress to deal with the questions involved, at Paris in 1915. The outbreak of war prevented the holding of that congress, as it also prevented the publication of this Memoir, which was written originally in 1914. Our paper deals with our own experience in anti-rabic treatment and gives the results of our statistical and experimental enquiries. It is intended as a preliminary contribution to discussion on the subject. Our own experiments prove protection in animals that have been immunized before infection. What we are examining into here is, not the efficacy of the treatment, but the degree of efficacy.

The Kasauli Pasteur Institute was the first of its kind in India. At the commencement of its history some 300 persons, European and Indian, were treated. That was the case in the year 1900-1901. Up to 1920 there have been treated 60,996 persons (Table I). The experience gained during these twenty years, in the treatment of rabies infection, is considerable. The modes of treatment have varied considerably. From the year 1900 the method of Pasteur, in which patients are inoculated with dried cords, was the method in use. The only variations from the orthodox Pasteurian procedure during this period were the utilization of slightly different gradings of dried cords and the conjunction in certain bad cases of a serum treatment with the vaccine treatment. At the end of 1907 the dilution method of Hoegyes was introduced by Major Lamb, I.M.S. This innovation was based upon the work done by Harvey and McKendrick on anti-rabic immunization (*Scientific Memoirs, Government of India, No. 30, 1907*) and was also rendered obligatory by the difficulty of obtaining a sufficiency of rabbits for the Pasteurian procedure. The third change was made in 1912 and was based upon the researches of Ferri (of Sassari) and of Sir David Sample (*Scientific Memoirs, Government of India, No. 44, 1911*). During the period of use of dried cords there were cases of nerve disturbance which occurred in patients during or after treatment. At the time these were very puzzling, although the

manifestation has now become well known to those administering anti-rabic treatment. These "accidents paralytiques," as they were called by the writer (Remlinger), who first drew definite attention to them, are fortunately rarely fatal and the paralyzes are usually completely recovered from. Remlinger (*Annal. Pasteur*, 1905, p. 625) described 40 cases out of 107,712 persons treated. They occur in very few instances, considering the enormous numbers of persons treated all over the world, and have been ascribed to the effect of a rabies toxin as distinguished from the rabies organism. Other factors than treatment must undoubtedly be concerned in the production of this unfortunate, though happily rare, accident. These have been set down as idiosyncrasy, alcohol, syphilis, fatigue, chill, etc., all representing in effect a lowered vitality of the patient, which would afford an opportunity for the action of a toxin. Harvey and McKendrick in their *Memoir* (1907) do not accept the view that this lesion is due to a rabies toxin, but regard it as more probably due to the action, direct or indirect, of the large amount of foreign nerve protein which is inoculated along with the rabies virus in Pasteurian inoculation. It was largely this view that led to the adoption of the dilution method of Hoegyes in the 2nd period of the history of the Kasauli Institute. With this method very minute quantities of foreign nerve material are injected as compared with the ordinary dried cord method. The safety of the method seemed assured from the very extensive experience of its use by the late Professor Hoegyes at the Buda Pesth Institute, an institute which at the time when the change was made at Kasauli was treating upwards of 4,000 persons annually and was, in regard to numbers treated, the largest institute in the world. After the dilution method was adopted the paralyzes which we have referred to in connection with the dried cord method of treatment ceased to occur.

The change to the use of a dead virus was made in 1912 as a result of the work on the subject, which showed that equally good protection was obtained by this method as from those previously in use. Much work of an experimental kind still remains to be done in regard to the keeping properties of the carbolyzed vaccine, its dosage and so on, but the important fact has been determined that it is as protective as the older living virus vaccines. We are enabled to make this statement not only on account of the considerable experimental work done by ourselves on the subject, but also because we now have available the result of 10 full years' use of the vaccine in routine treatment.

Up to the year 1912 the practice at Kasauli and elsewhere had all been in the direction of magnifying the risk of infection. Authoritative writers all give prominence to that risk. Thus Masini (Bull. Institut Pasteur, Volume 3, 1905, p. 788) reviewing Rendinger's work, writes:—

'These results show the danger of bites which would be considered insignificant, and of the licks of suspected animals on apparently healthy surfaces.'

We ourselves, however, began to consider much more attentively the question of degree of exposure to risk and probabilities of infection. This necessitated a thorough investigation of the current ideas on the subject of the mortality rates of rabies, such points as incidence of the disease in those exposed to risk, the true comparison of rates amongst the treated and the untreated, the correction factors to be applied to different populations, and many other points which will duly emerge from the discussion of those rates upon which we now enter.

## 1·2 MORTALITY RATE OF RABIES.

**1·21 DEFINITION.**—The mortality rate of an ordinary disease is a well-understood measure of its severity. The number of deaths amongst those contracting the disease can be computed with greater or less exactness. In the case of rabies we may affirm, with but little danger of contradiction, that in this sense the mortality rate is 100 per cent. Once the disease has declared itself in man there is no question of what will be the issue: the issue is death. In all reports of anti-rabic institutions death rates of the treated are given. These are necessary in the first place as evidence for the efficacy of inoculation in the treated as compared with the untreated, and in the second place for the purpose of comparison of the results obtained with those of other institutes. It is then in connection with the appraisement of the value of treatment that the mortality rate is particularly required. But we are at once confronted with great difficulties in our computations. We are dealing with a disease of very great extremes of incubation as is evident from an inspection of Tables VIII and IX. The different methods of treatment of the disease differ greatly from each other as regards duration of application. Again the time at which a patient appears for treatment is very variable, as regards the interval which has elapsed since he was supposed to have become infected. These are factors which must have the most important bearing upon the efficacy of the treatment and its power to protect from death. If we simply take an ordinary death-rate with reference to a population in it

presents itself for treatment at the Institute we may find that we come to be 'treating' a patient within a few days of his developing the disease. Under such circumstances it can hardly be said that there has been any treatment at all. But where shall we draw the line between what is treatment and what is not treatment? That is the difficulty. Again a patient who starts his treatment one or two months after his exposure to the risk of infection is scarcely to be claimed as a 'cure' for the treatment, considering that by the time he comes under treatment the best part of any risk he originally ran is obviously and rapidly becoming negligible. When we take all these and other circumstances into account it seems advisable to adopt a simple death-rate as our basis of comparison of the results of different institutes of different methods of treatment. Such a death-rate would be given by number of persons dying in spite of treatment out of all those who have actually applied for treatment. The adoption of this rate may tell hardly on the results of treatment in certain instances, but if we assume, or are aware, that very much the same habits prevail as regards presentation of persons for treatment in different parts of the world, we become enabled to institute comparisons of results on an equal basis. This is not at all the case for the so-called 'failure rate,' which, although reasonable enough in some respects, should, and yet does not, take account of duration of treatment and other circumstances which affect it markedly. This question will be discussed in detail presently.

**1·22 CORRECTION OF DEATH-RATES.**—It is usual when comparing death-rates in any disease to make proper corrections of the rates for purposes of comparison. Such corrections have reference, for example, to age, sex, occupation, and so on. Where we are dealing with a disease such as rabies and making comparison of the results of treatment we ought to demand the application of a very much larger number of corrections than are even applied in the case of ordinary death-rates. We should require corrections for race, severity of wounding, lateness of arrival, duration of treatment and others, before we can have comparisons such as are being constantly made. Then again when we examine into death-rates in the treated and in the untreated we require even more evidence than is needed for the comparison of results obtained in treatment only, before we can admit that the rates given are really valid for comparison. It is certain that the number of deaths set down as occurring amongst the untreated is a figure of very doubtful accuracy. The great tendency in the case of statistics of the untreated is to weight the data with deaths and undervalue the population at risk. The result is

to produce a greatly exaggerated death rate amongst the untreated. What is badly needed then at present is correction of death rates, in the first place, for the treated of different institutions and, in the second place, for the untreated populations when compared with the treated. For such corrections to be made it is essential that we should have standard populations with well-defined characteristics of the kind which matter in a disease like rabies. When we have such a 'Standard population' then we can make the necessary reductions by means of proper 'correction factors.' Such a standard population could be obtained from the data which should be given by Pasteur Institutes. Unfortunately the data are conspicuous by their absence in the ordinary reports. We consider that the data given since the year 1911 in the Kasauli reports would afford material for the construction of a standard population (treated). The data required are such as severity of wounding (depth of wound, locality of wound, interposition of clothes, number of wounds, etc.), lateness of arrival, duration of treatment, age, status of patient and so on. When, however, it comes to a comparison of treated with untreated the difficulties in the way of proper correction of rates seem almost insurmountable.

**1'23 DIMINUTION OF MORTALITY RATES WITH CONTINUED EXISTENCE OF INSTITUTES.**—Mortality rates often tend automatically to become lower and lower with the continued existence of a Pasteur Institute. This is partly due to the inclusion of larger numbers in the totals of the not-at-risk in successive years. At Kasauli the error is not so great amongst Indians as amongst Europeans, for the Indian is much less disposed than is the European to present himself at a Pasteur Institute for treatment, unless there is reasonable probability that he has been bitten by a rabid dog, and unless he suffers from something definite in the way of a lesion. As regards definiteness of lesion the statement is obvious from a comparison of degree of wounding of Europeans and Indians, respectively, attending for treatment, as given in Table III.

**1'24 ORIENTAL POPULATION MORE EXPOSED TO RISK THAN EUROPEAN.**—Remlinger draws attention to the higher mortality rates which obtain in Constantinople than elsewhere. The explanation which he gives of the fact is that the people treated there are more severely bitten, arrive later, and are more frequently bitten or have skin or through thin clothing than those of colder countries. The case is exactly the same for the Indian and he is distinguished in these reports

from the European in India. In fact an Oriental population is more truly exposed to risk than a European one and is therefore a better one to take than the latter for statistical purposes. Our own Table III confirms Remlinger's view and the whole statement is in accordance with our experience in India.

**1·25 EFFECT OF LATENESS OF ARRIVAL ON MORTALITY RATE (TABLES II AND VII).**—The percentage mortalities by weeks are, for the first four weeks (Table VII):—

Arrival within 1st week 2·9 per cent.

..	..	2nd	..	1·3	..
..	..	3rd	..	2·2	..
..	..	4th	..	2·6	..

These are irregular percentages and the irregularity may have to do with the proportionate degree of risk run by individuals who arrive in certain weeks, or to an insufficiency of totals to give statistical smoothness.

McKendrick, *Indian Journal of Medical Research*, Volume 5, 1918, gives the following analysis of very extensive data showing mortality for locality of wounds and lateness of arrival:—

Arrival during				Percentage mortality amongst persons bitten on	
				Arm	Face
1st week	..	..	..	0·94	6·64
2nd	..	..	..	1·47	9·09
3rd	..	..	..	1·94	10·64
4th	..	..	..	0	14·29
5th	..	..	..	2·15	12·50
6th	..	..	..	0	0

The patients who come to a Pasteur Institute come at very various intervals of time after they have been bitten: some arrive so late that they are well into the ordinary incubation period of rabies before they even begin treatment. It is not to be wondered at therefore that some of these cases die during their course of treatment or very shortly afterwards. What sometimes happens, in Indians at all events, is that persons bitten do not make a move in the direction of an Institute until the occurrence of a death amongst their numbers. Such as do so then must obviously be in a very different position from those who come up in the ordinary way for treatment very soon after being bitten. To begin with, if they are really infected, they are well through their incubation period and will in that case be likely to develop the disease during or very

shortly after treatment. By the convention which gives us the so-called 'failure rate' the onset of rabies during the course of treatment, or within fifteen days after its completion, entitles us to exclude such cases from the 'failures of treatment.' The case is in fact regarded as if it had not been treated at all. On the other hand, if death does not occur, the patient is included in the totals of the cured and treated. Such a procedure, even if to some extent justified by the argument as to what constitutes a failure, does not conduce to the comparability of mortality rates.

### 1·26 SEVERITY OF WOUNDING AND MORTALITY RATES. —

As a character which may be regarded as the main contribution to severity of wounding we may take number of wounds. Table VII shows the distribution of wounds among non-fatal cases (Indians) and fatal cases at Kasauli in 1911. All fatal cases which are included in this table are not differentiated in any way as failures or non-failures. This is done throughout this memoir unless otherwise stated. In compiling the tables, only those cases were included where the information was definite. The few cases in which the number of wounds was described as 'several,' 'multiple,' etc., have been excluded.

There can be no doubt that number of wounds is one of the most certain factors, though not the only one, in the production of infection. It is however very difficult, on the basis of one character or for the matter of that any assemblage of characters, to be certain that infection has even taken place. The saliva of a rabid dog need not necessarily be infective, need not necessarily contain the virus at all. This is a point of view the possibility of which, as far as we know, had not been insisted upon until the publication of the work of Crumshank and Wright (*The Indian Journal of Medical Research*, Vol. 1, No. 4, 1914, p. 741) on the experimental transmission of rabies through the inoculation of saliva.

### 1·27 MORTALITY RATES AMONG TREATED AND UNTREATED. —

We wished to obtain data which should be free, if possible, from the objection of non-comparability and of being weighted with deaths. These we endeavoured to collect by following up the cases of persons bitten but not presenting themselves at the Institute for treatment. The information regarding such cases was obtained from our patients in the first instance. Our procedure then was to write and ask the officials, especially the Civil Surgeon of the district concerned, for the numbers of persons who had been bitten at the same time and by the

same animal as any given patient who had come for treatment but who themselves did not come for treatment. These enquiries were continued for three months after the information was obtained, which is the same period of supervision exercised over treated patients. We were thus able to collect a number of such cases. The comparison made here of the untreated was with such persons as had been bitten by the same animal and at the same time. This implies that more than one person must have been bitten and consequently that there was considerable probability in all cases that the biting animal was rabid. In the population of the untreated (Table XVII) the percentage mortality rate was 10·7, a lower figure than that of Hoegyes, which is the one usually quoted, and is 15 per cent. In the calculation of our percentage we have included practically all deaths which occurred, although many of the deaths may have been due to causes other than rabies. Even in the case of these data we consider that the rate is unduly weighted with deaths, not only deaths from diseases not rabies, but also with deaths from rabies. This is due to the undoubted tendency of any reporting agency to pay special attention to the occurrence of deaths and their reporting these, and to failure to obtain the totals at risk. There are some very various estimates of the mortality amongst the treated and untreated. Almost all pitch the rate too high. We may consider some of these estimates. The following statement is made by Marx (art. *Lyssa*' Handbook d. path. Mikr. Kolle and Wassermann, 1st Ed., Vol. 4, No. 2, p. 1269): Hoegyes supplies the following data from Hungary. In the period from 1890 to 1895 there died, out of 985 persons bitten who had not undergone treatment, 14·94 per cent. Hoegyes from these figures takes the average number of deaths to be 15 to 16 per cent, and again:—'Kirchner in Germany has perhaps reckoned the deaths at their lowest. In the period from 1st January, 1891, to December 31st, 1901, 1,453 persons were bitten by rabid or suspected rabid dogs. Of these 38, 2·32 per cent died of rabies. This figure differs completely from all others. As, however, until a short time before the completion of this period, no necessity existed to report deaths from rabies in cases of persons bitten by rabid animals, we cannot accept the statistics as entirely unobjectionable. The statistics however show that on the whole the mortality in Prussia is lower than the figures given by Hoegyes. If we accept a mid-value, then we may say that 6 to 10 per cent probably represents the true mortality amongst the untreated.'

Babes (*Traité de la Rage*, 1912, p. 602), commenting on statements as to mortality in the untreated, makes the pronouncement 'The great majority of authors give 10 to 20 per cent as the proportion of untreated persons who die from rabies if bitten by a rabid dog. Romlinger adopts the intermediate figure of 15 per cent. I myself, when I think of the large number of persons bitten, who are not known whilst cases of death from rabies are well known, and when I compare the statistics of bites before and after the institution of Pasteurian treatment, am persuaded that even the figure 10 per cent is exaggerated and that it is nearer 5 per cent of persons bitten by rabid dogs and not treated.' In another place (*Zeitsch. Hyg.*, Vol. 58, 1908, p. 401) Babes states: 'The majority of authors are of opinion that in taking 50 per cent as the proportion of the bitten who are bitten by really rabid dogs we are not overrating the case.'

Here then we have some considerable diversity of opinion as to the real mortality amongst the untreated. It will be worth our while to examine the question in more detail.

We shall take Hoogyes' figures. These have been generally regarded as a reasonable estimate, although it will be evident from some of the quotations given above that there is nowadays a tendency to believe that they form an overstatement (cf. Kirchner, Marx, Babes). The importance of Hoogyes' figures consists in this that they were specially and officially collected. We know of no other statistics quoted in the literature on rabies which are quite on the same footing as regards the purpose for which they were collected.

The following is our summary of the statement and interpretation of the figures given by Hoogyes (*Nothnagel's Handbook "Lyssa,"* pp. 168-169): From 15th April to 31st December, 1895, there were officially reported in Hungary over 5,899 cases of bite from presumably rabid animals and of these 985 were untreated. Of the 4,914 treated 106 died, made up of (1) 59 dying more than 15 days after completion of treatment, 'failures,' and (2) 47 dying during treatment or within 15 days of its completion. This figure 106 gives a mortality rate of 2.1 per cent for the treated, but, owing to the convention as to what constitutes a failure of treatment, the 47 who died during treatment or within 15 days of its completion are excluded by Hoogyes from the computation; we are left therefore with 59 deaths amongst the treated out of 4,914 cases, or a mortality of 1.2 per cent. Further Hoogyes, not content with excluding by means of a convention 47 deaths from the class of the treated actually

seems inclined to add them to the untreated ( $985 = 938 + 47$ ). In this way he obtains a mortality amongst the 'untreated' of 14·9 per cent (147 deaths in 985). The subtraction of a certain number of the deaths from the total number of cases dying after having undergone a partial or a complete course of treatment is a method of computation based entirely on the conception of what is called a 'non-failure' of treatment. This, in its crude form, is surely a very unsound method of calculation. Not only is the method of calculation of the mortality rate unsound, but we are not supplied with even the most elementary consideration of the constitution of the two populations compared. We have no data as to the severity of wounding of the untreated population, no estimate of the number at risk in either treated or untreated populations. There are other objections to these statistics with which we shall deal later. Most important of all is the objection that statistics of mortality amongst the untreated are largely overweighted with deaths. This fact together with the certainty that the totals amongst the treated contain a considerable number of the not-at-risk are of themselves sufficient to cause considerable divergence in the mortality rates of the two populations. Such a divergence is due to fallacious reasoning as to comparability. Kirchner's figure 2·32 per cent is based upon the following statement (Kirchner *Klin. Jahrb.*, 10-2-'02, p. 178). 'In the period from 1st January, 1891, to 31st December, 1901, 1,453 persons were bitten in Prussia by rabid or suspected rabid animals: of these 38, 2·32 per cent died of hydrophobia. These deaths were distributed as follows in the single years (see Table XI).

Marx (Handbook d. Path. Mikr. Kolle and Wassermann, 1st Ed., Vol. 4, No. 2) seems to assume that the figure 2·32 per cent represents the mortality of the *untreated*, but Kirchner nowhere says that they were untreated. In fact, although previous to 1898 the probabilities are that very few of those recorded as bitten were treated, nevertheless in the years subsequent to 1898 most of them were treated. But even if we take the percentage mortality for the years 1891 to 1897 only, we still find it to be very much lower (only 3·8 per cent) than that given by Hoegyes. One thing which strikes us forcibly in Kirchner's table is the marked reduction in percentage mortality immediately after the establishment of the Prussian Pasteur Institute in 1898. We are left in doubt, however, in so far as this table goes, whether the fall in percentage mortality from rabies may not be partly due to the introduction of many individuals in the total bitten who, before the establishment of an

Institute, could never have reported themselves as bitten. The numbers reported bitten go up immediately with the establishment of an institute, but we are compelled to admit from our own experience that a certain number of these must be classed as being not at risk individuals who previously did not report themselves at all.

But Kirchner does give an actual estimate of the difference in mortality between treated and untreated. His rates for each class are both of them low: 0.15 per cent for the treated and 3.5 per cent for the untreated.

Treated.	Deaths.	Rate.	Untreated.	Deaths.	Rate.
652	1	0.15%	483	17	3.5%

We are not told anything about the series. Kirchner's previous table shows 18 deaths between 1897 and 1901. Are we then to take it that all the 17 deaths which occurred in these years were untreated and only one treated? Or has Kirchner done what Heegves did, and classed all deaths among treated persons, which were not 'failures' of treatment, as untreated?

The important point, however, for our discussion is that Kirchner's rate for the untreated 3.5 per cent is an unusually low one. Before we could decide which of these two rates (15 per cent Heegves or 3.5 per cent Kirchner) should be accepted as the true one for the untreated, we ought to know what was the mode of collection of the figures and what was the constitution of the population on which the percentages are based. Unfortunately this information is not given. Marx gets over the difficulty of the divergence between these two authorities by taking a middle value and regarding the mortality among the untreated as being about 6 to 10 per cent.

Doebert (Klin. Jahrb., Vol. 21, No. 1, 1909, p. 34) makes a calculation of the probable number of deaths in 6 years which would have occurred if treatment had not been adopted. Not 10 deaths (the actual), but 275 deaths would have occurred. Here again, we have what must be an overstatement of the benefit derived from anti-rake treatment. Such overstatements are in the long run calculated to do the cause of treatment more harm than good. Doebert might have found, on consultation of the Prussian statistics (Table XI), that the total number

of deaths, for a period of not less than 30 years (1886 to 1897) antecedent to the establishment of any anti-rabic institute in Prussia, appears not to have exceeded 220 in all.

Babes' statement, a very moderate one of a 5 per cent mortality, seems to be based on general considerations, especially this that the ordinarily given statistics pitch the death-rate much too high. He says, for example (*Traité de la Rage*, 1912, p. 38): 'The more carefully statistics are collected, the smaller does the death rate become in comparison with the number of persons bitten; that is to say, it is much easier to recognise cases of hydrophobia and of death than to record cases of bite.' Again on page 40 (*loc. cit.*) he says: 'The death rate would appear to fall in proportion as the numbers bitten increase; such statistics would nowadays be regarded with grave suspicion. Very often the bites are inflicted by non-rabic dogs. Another source of error in the statistics is the very variable gravity of the bite inflicted: it is evident, therefore, that we do not possess any sufficiently good data to enable us to appreciate what is the relation between mortality and numbers bitten.'

These quotations and the record of our own investigations on the subject show how difficult it is to form an estimate of the mortality rates amongst the untreated with which to compare those amongst the treated.

**1·28 CONSTITUTION OF POPULATION AND MORTALITY RATES.**—It will be evident from the remarks made here and there in what has preceded that the constitution of the population concerned will have the greatest effect on the mortality rates for that population. This subject of the constitution of population has scarcely received the attention it deserves. We may consider the general question under the particular heads of:—

- (1) Mode of collection of statistics.
- (2) Degree of exposure to risk.

(1) Mode of collection of statistics.—This is a perfectly straightforward matter as regards the treated. The total numbers appearing at an institute for treatment with details of the circumstances of their case is fully known and is the total treated. The case for the untreated is very different: it must be very difficult indeed to get any proper totals in this case at all. If the collection is made through public dispensaries or through medical men, it is obvious that only such cases as have wounds of sufficient importance to require medical aid will be reported at all. But very many cases of much less

degree of wounding than thus go into the totals of a Pasteur Institute population. If, again, the information as to the untreated population is obtained with the assistance of the veterinary service of the country, the total will still show deficiency of numbers, in the first place because of the number of rabid animals dying unmedicated, and in the second place owing to the refusal of some persons to admit, having been bitten at all, much less only licked. Part of the information as to numbers of the untreated population may be obtained through patients attending at a Pasteur Institute, but such information depends on the willingness and accuracy of such patients and leaves out of account such cases as do not supply out of their number at least one person who has resorted to Pasteurian treatment. No such limitations cause impoverishment of the totals of the treated populations. But we believe that there is another reason altogether which is responsible for the high mortality rate amongst the untreated. It is that the collection of statistics of the untreated is made largely on the basis of the occurrence of deaths from hydrophobia. Registration of deaths and their causes has been in force in most civilized countries now for a considerable time, even although special compulsion to report cases of death from rabies in particular may not have existed. Every case of death from rabies is seized upon as bearing upon the mortality rate. If it is a treated case, it will be already known, at least with regard to number of bites and other details: if it is an untreated case, it will be added to the mortality of the untreated. But we doubt very much whether special steps have been taken to accurately ascertain the total numbers bitten on the same occasion as an untreated death and themselves untreated, or the numbers bitten, untreated, and not supplying a death at all. The latter total must be almost impossible to collect satisfactorily. If the method here commented upon, of collecting statistics of the untreated largely on the basis of the occurrence of deaths amongst them, is the one followed in the compilation of statistics of mortality in the untreated, then there has undoubtedly been overweighting of that population (the untreated) with an undue proportion of deaths. That such a method has been followed, and followed without the least idea of its consequences, is evident from Hooghyes' procedure of passing over all cases of death which were not 'failures' into the class of the untreated. Further, if the collection of statistics of the untreated has been made on a basis of occurrence of deaths, then we may say at once that the population concerned is largely, if indeed almost wholly, one at risk. Such a risk, however, also runs for the treated

population as is evident from Babes' statement regarding the proportion of the treated who are bitten by probably rabid animals. There is also a very large inclusion in the population of the treated of those who are not-at-risk or under diminished risk for various reasons not connected with the rabidity or non-rabidity of the animal concerned, cases of slight contact with saliva, late arrivals, etc. This aspect of the case is dealt with in the next paragraph.

(2) Degree of exposure to risk.—We may preface this subject by saying that the degree of risk, even in the case of definite bites by a certainly rabid dog, is not as great as is often imagined. If this were not so, rabies would be much more common than it is. Even old authors recognized this fact, although it seems to have become obscured for a time: Forthergill ('Works' 1783, Vol. 2, p. 241) says: 'Notwithstanding these discouragements there is one thing which ought to afford the sufferers some consolation, which is that it appears very evident that, if no means of prevention were used, many of those who are bit by mad animals would never be liable to the fatal consequences of canine madness.' The majority of authors, according to Babes, consider that about 50 per cent at least of persons attending a Pasteur Institute are bitten by non-rabid dogs (*Zeitschf. Hyg.*, Vol. 58, 1908, p. 401). Then again persons who are only trivially bitten, or cases of merely salivary contact, will not report themselves and will not seek medical aid, and will not therefore, if untreated, be included in the totals. But Pasteur Institutes gather all and sundry into their totals and in course of time attract such persons as would otherwise pay no heed to their condition or consider that any treatment was requisite for them. We have among Pasteur Institute cases persons bitten through clothing, with skin unbroken, persons not bitten but slightly bruised, persons merely licked by dogs, persons who have administered physic to mad dogs, persons who have been in contact with mad dogs, persons who have picked up articles which have fallen from the mouths of mad dogs or handled their chains, attendants on hydrophobia patients, etc., a long list which might be extended, a list which seems to become all the longer the longer the institute has been in existence. Thus it comes about, especially where the educational influence of a Pasteur Institute has made itself thoroughly felt throughout the land, that a very large proportion of the treated cases are cases which are excluded from an untreated population and which are often only to a slight degree exposed to risk.

If results of Pasteur Institutes are to be compared with each other, still more if their mortality rates are to be compared with rates amongst the untreated, then the constitution of the several populations as regards degree of exposure to risk should be very closely attended to. Tables II to VII illustrate what we mean by our insistence on the necessity of full details as regards the factors which determine degree of risk. These tables give, we consider, most of the characters required on which to form an estimate of risk and also to enable us to obtain a good idea of the type of case which is being treated at any given institute. With such data 'corrections' can be applied to crude death rates and the corrected rates compared with each other. At any given institute, such as that at Kasauli, we are enabled by means of such tables as these to compare the type of case treated from one year to another and to note whether changes are taking place in the constitution of the population in attendance. We can see at a glance what marked differences there are in severity of wounding between Europeans and Indians and so insist on the necessity for separation of these two sub-populations. We can determine how many persons in the total submitted to treatment are merely licked (recorded as having 0 wounds), how many bitten on bare skin, how many cauterized, how many suffering from multiple wounds, how many late in arriving (*i.e.*, far advanced into the incubation period of rabies) and so on. All these facts are of the utmost importance in gauging the type of population coming under treatment at a Pasteur Institute and so of great importance for the estimation of the results obtained. But, with the exception in some cases of information regarding locality of wounds, absence of clothing and cauterization, no information is forthcoming on these points in the reports of Pasteur Institutes. A very much fuller and more extended set of tables than ours has been given by McKendrick (Quinquennium, 1912 to 1916): these were rendered possible by the accumulation of the data (*Ind. Journ. Med. Res.*, Vol. 5, No. 2, 1917, p. 413) which went to make up our tables.

#### 1.29 INCUBATION PERIOD OF RABIES AND THE MORTALITY RATE.

(1) Incubation period after subcutaneous inoculation of Street Virus in the rabbit.—Much of the experimentation on this point has, if we mistake not, been done with rabies brains preserved in glycerine for a longer or shorter time. It has been assumed that the glycerine has not produced any effect on the virus at all, at least during the ordinary time of preservation. We do not think this assumption justified. This conception of an average survival

of 15 days' incubation is taken for granted by practically all institutes and is reflected in their statements as to their 'failure rate.' A term which has of late years been constantly used in the literature on rabies is 'virus renforcé' as descriptive of certain street viruses in which the incubation period is much earlier than the average 15 days. But as there exist very considerable variations from this average, and there is no reason to suppose that the variation is anything but continuous, we see no justification for the use of the term. The frequency distribution taken from our own experience shows what these variations may amount to for rabies brains, practically all of which were sent to the Institute from a distance, and in glycerine, see Table XVIII. We see from the Kasauli table that in the majority of cases the incubation period is 11 days and not 15 days and that a considerable number of cases show periods of 8, 9, 10 days and so on. We are disposed to think that even the average incubation would be less than 15 days for brains freshly taken from dogs, killed on account of rabies, and that the great prolongation shown in the table and chart may be simply due to the rabicidal effect of glycerine, combined with, in certain instances, the effect of high temperature during transit.

(2) Incubation Period of Rabies in Man.—The table which is most frequently quoted is that of Bauer (Muench. Med. Woch. Nos. 37, 39, 1886). He found a mean incubation period of 126 days in 537 cases. When 10 doubtful cases are extracted, and 17 where the period of incubation has been more than  $1\frac{1}{2}$  years, we obtain a mean incubation for 510 cases of 72 days and a distribution as follows:—

No. of days				Percentage.
1 to 19	..	..	..	8.24
20 to 39	..	..	..	28.34
40 to 59	..	..	..	21.18
60 to 79	..	..	..	15.30
80 to 99	..	..	..	9.22
100 to 149	..	..	..	7.65
150 to 199	..	..	..	5.69
200 to 249	..	..	..	0.98
250 to 339	..	..	..	2.35
12 months to 15 months	..	..	..	1.18

In Table XX incubation periods vary considerably. Hongyus' and Nitsch's data (Table IX) show that the treated population has a somewhat shorter incubation period than the untreated one, due in all probability to the inclusion of greater numbers of the badly wounded in the treated population. We may take Bauer's table as reasonably correct. We see that some 37 per cent of persons dying die within 40 days from the date of bite. The importance of this and the similar facts set forth in the table will become apparent when we treat in more detail of the meaning of 'failure of treatment.'

**1·3 : 1·31 MORTALITY RATES, RABIES.** Having so far considered the effect of various factors and circumstances on the mortality rates of rabies we are now in a position to consider the rates themselves. There are two rates in use at Pasteur Institutes. Sometimes the one and sometimes the other is taken to describe the mortality after treatment and it is not always stated which is meant. The total mortality rate is simply the percentage of persons dying in spite of treatment and is quite easily understood. The failure rate or the percentage of persons in whom treatment has truly failed to establish protection requires for its understanding a knowledge of what is regarded as a failure of treatment. Only those persons are accounted 'failures' of treatment who die later than 15 days after the completion of treatment. By this definition far the greater proportion of deaths, amounting to as many as 80 per cent of the total deaths (Table XV), may be excluded from the failures. We must examine into the soundness of the conception of what constitutes *failure*.

The idea of failure is based on the fact that when street virus is inoculated subdermally into a rabbit the symptoms in that animal appear first, on an average, in 15 days. It is laid down further that, if the virus once reaches the central nervous system, no treatment can be of any avail against development of the disease. The disease will inevitably appear in some 15 days after the arrival of the virus there, therefore it is argued that, if the virus has not been prevented from reaching the brain by the time of the last inoculation, it will not be and could not have been prevented from producing the disease. The argument is a subtle one and the underlying idea is evidently that the full effect of the complete treatment is required to produce that amount of immunity which will be capable of coping with the infection while it is still peripheral. This then is the idea which

enters into the conception of failure (*insuccès réel*). Besides being the average interval to the manifestation of symptoms of rabies after the virus has reached the central nervous system, this period of fifteen days is also regarded as being necessary to the development of maximum protection after the last inoculation has been given (*Babes' Traité de la Rage*, p. 589). The views held by different authors, however, differ considerably on this point. We have seen also how the average period of fifteen days, after subdural inoculation of street virus in the rabbit, is merely an average. It is even doubtful if it is the period of maximum frequency. There are many differences from the average both on the side of defect and of excess. Where the failure rate fails entirely, in our opinion, for comparative purposes is in so far as no account is taken in reports of Pasteur Institutes of the current practice of treatment of late arrivals, and the duration of treatment. Both these factors, lateness of arrival and duration of treatment have a most marked effect upon what the 'failure rate' is going to be. For this reason we advocate the use of the total mortality rate for purposes of comparison. Our contention is that Pasteur Institute are able to show diminution of mortality simply amongst those who present themselves for treatment and begin it, as compared with those who do not, without any definition of what actually constitutes treatment. The limitations of time imposed by the definition of failure are from some points of view objectionable and might be discarded without any great disadvantage accruing. Some institutes go the length of publishing only the figures for failures, as the only proper deaths from their point of view. Now there are two periods which go to make up the interval within which a Pasteur Institute death is not considered a failure. They are:—

- (1) Deaths occurring during the course of treatment.
- (2) Deaths occurring within 15 days after the completion of treatment.

A long interval may however elapse before a patient even arrives at an Institute for treatment and the result may be, naturally enough, death during treatment. It is obvious then that we might go the length of saying that the later a patient arrives for treatment the less likely he is to be a failure of the treatment should hydrophobia supervene. If he come late and die, he will probably fall into one of the non-failure periods. If he does not die, he will go to swell the total of cures on which the failure rate is calculated. But the possibility of placing

a rather too favourable interpretation on the results achieved by treatment does not end here. The failure rate is lowered by increasing the duration of treatment. This may vary greatly according to the Institute, from about 10 up to as much as 40 days. Thus it is not difficult to see one possible explanation of why an institute giving, say, a 28 to 30-day course should be able to show a zero failure rate, whereas one with only a 14 day course is unable to show anything like this success.

Finally, we add to the interval before arrival and the interval of application of treatment a third interval, a constant period of 15 days to make up the full period within which a case of death does not count as a failure. A person who arrived at an Institute seven days after being bitten and received an 18 days' course (at one time the ordinary Kasauli course) could not possibly be regarded as a failure until the expiry of 40 days ( $7 + 18 + 15 = 40$ ) after the date of his infection. The meaning of the remark that, as shown by Bauer's table, 37 per cent of the untreated die in any case within 40 days of the date of bite will now be apparent. Remlinger publishes a very valuable table (Table XV) of cases treated at Constantinople during the years 1901 to 1908. Out of 6,808 persons treated, there were 99 deaths, but of these only 19 were failures of treatment; that is to say, he is able by means of this convention to exclude 80 out of 99 deaths from his computation of a failure rate. Hongves not only considered such cases as of no consequence, but made them over to the class of the untreated. It will be obvious, in the light of these remarks, why we prefer the total mortality rate as a means of gauging the efficacy of a given mode of treatment and the success of a given institute. The total mortality rate itself of course should have appropriate corrections applied to it in its crude form.

Table XII gives the results obtained at various Institutes at one time or another. Some of the percentages here given undoubtedly refer to total mortality but most of them represent the failure rate. The variations, with few exceptions, are not great even as they stand and if the correction factors for constitution of population were applied to them, the variation would presumably become still less. In regarding the consideration of mortality rates from the point of view of treatment or no treatment, we have set out in tabular form the causes which we consider account for the divergence of the currently accepted mortality rates from the true rate.

*Points Explanatory of the Divergence of Mortality Rates amongst the Treated and Untreated, other than Treatment itself.*

Treated.	Untreated.
1. Inclusion in the totals of the little or non-exposed to risk :—(non-rabid animal, trivial wound, late arrival, etc.)	1. Little or non-exposed to risk are largely excluded, because not reported.
2. Totals complete.	2. Totals incomplete.
3. Totals bitten and treated for a given death complete.	3. Total numbers bitten and untreated corresponding to given deaths incomplete; often the death is the only untreated case reported.
4. Adoption of a purely conventional death rate (failure rate), instead of rate given by total mortality.	4. Retention of rate given by total mortality, and non-adoption of any convention.
5. Subtraction of deaths not ranking as properly treated, <i>i.e.</i> , non-failures.	5. Addition of deaths not ranking as properly treated to the <i>untreated</i> .

The conditions here enumerated as causing this divergence of rates are not all mutually exclusive. The table is given chiefly for the reason that it is a summary of the argument which precedes it. So far we have considered mortality rates from the point of view of treatment. It is important however to look into prophylactic measures other than the treatment of human beings, which are of assistance in eradicating this disease. Now rabies is a disease of which we may regard the dog as being the transmitter to man. There are a few other animals concerned but they are of very little importance as compared with the dog. If we desire to cut off the source of infection, our efforts to do so must be directed against the dog. It is natural then that we should find that some countries have paid special attention to this point. What has been their success?

**1·4 LEGAL ENACTMENTS AND RABIES MORTALITY.**—One of the most notable examples of a successful victory over the disease brought about by restrictive measures is that of Great Britain. Other countries which have no rabies are Norway, Sweden, Denmark, and Australia. Germany, and especially Prussia, has little or no rabies and

the regulations with regard to control of rabies in dogs have been well enforced in Prussia. Babes (*Traité de la Rage*, 1912, p. 30) says:—

'In Germany there are half as many rabid animals as in France. The relation between the number of rabid animals and that of infected persons is noticeably very small in Germany; especially in the centre it is to be noted that as a consequence of severe police measures there are only very few cases of rabies even in animals.'

In the case of Great Britain the relation of legal enactments to disappearance of rabies is one of the most striking instances of the conquest of disease that there is. Other countries, whilst not having quite the same success as Great Britain have had their legal measures. The year 1910 saw the mortality (failure) rate at the Paris Pasteur Institute reduced for the first time to zero. Previous to this year there had commenced specially vigorous measures against rabies in dogs and particularly in Paris in the Department of the Seine. Now with the establishment of other institutes in France and all over the world the Paris institute has come to be, in the matter of amount of anti-rabic treatment done there, more and more of a local institute. Local measures such as those referred to would naturally have their effect upon the clientèle of the institute and this is probably the reason for the attainment of the zero mortality. The following note taken from the *Lancet* of 19-11-10, page 1504, is interesting in connection with the attainment of the zero mortality at the Paris Institute in 1910. We ourselves have added the mortality rates for the years commented on to the table (XIV) given.

*Lancet Note*:—Report of the Veterinary Sanitary Service of Paris and the Department of the Seine, 1909. In England and Sweden rabies has been stamped out. Dr. H. Martel, Chief of the Veterinary Service and author of the report, considers that the most useful measure is to destroy all stray dogs. This of late years has been done vigorously in Paris and the surrounding department of the Seine, but not so in the other departments or countries of the Seine. These figures (table XIV) are based on the notifications made by Veterinary officers as the result of diagnosis or post mortem examination. As for the human cases of rabies, they are extremely rare in the department of the Seine, the last one observed dating back to the year 1905. Formerly they were relatively frequent. Dr. Martel insists that the great improvement brought about is due to care taken when a case of rabies occurs to destroy or in any case to closely observe all the other dogs it may have infected. As however

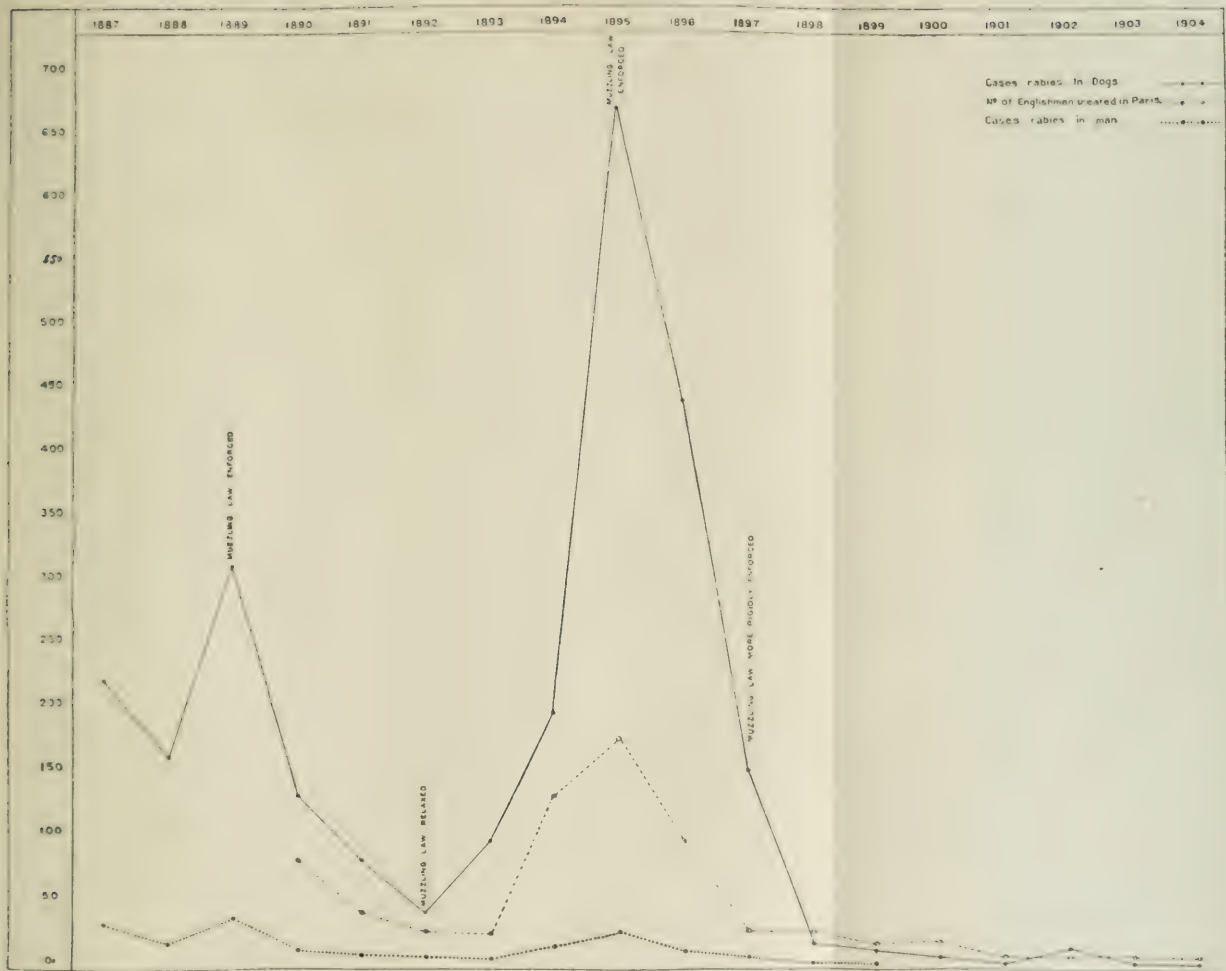
this can rarely be done in a complete manner, obviously the most effective measure is the capture of all stray dogs.

The way in which not only deaths from rabies in man but also the numbers requiring treatment on account of rabid dog bite follow the curve of rabies in dogs is very strikingly shown in Chart I for Great Britain. The final disappearance of rabies in animals and the attainment of a zero incidence in man is reached for Great Britain subsequent to the stricter enforcement of the control of dogs in 1897.

We also give a chart for Germany (Chart II). This shows a very striking resemblance to the chart for Great Britain. Whereas the British chart is of numbers of actually rabid dogs, that for Germany is of numbers of dogs killed either on suspicion of rabies or as ownerless. If we take into account the difference between the facts which they show, the similarity of form and the correspondence in time of the two charts is rather remarkable. It probably indicates correspondence of activity in the campaign against dogs in both countries. Can we show anything similar in Germany as regards association between legal enactments and diminution in rabies, as we have found for Great Britain? The first important law on the subject in Germany (*Das Reichsgesetz betr. die Abwehr und Unterdrückung von Viehseuchen*) was promulgated 23rd June 1880, and followed by 'Instructions' in February, 1881. A reference to table II which of course is only for Prussia shews that already by 1882 a distinct diminution in the human mortality for rabies had occurred and this continues thereafter. Another law came into force on 27th June, 1895, but we can discern no further diminution of mortality which can possibly be attributed to this law. Germany is not like Great Britain, an island which is easy to cordon. Countries in which legal restrictions on the keeping of dogs are in no way so severe surround Germany and rabid dogs pass into German territory over the frontiers. The geographical distribution of rabies in Germany bears out the supposition that cases which continued to show themselves in that country were a residue largely due to importation of rabies, which legislation cannot affect. This may possibly explain what seems to have been the success of the law of 1880-1881 and the want of further success of the law of 1895. We have however sufficiently demonstrated that legal enactments taken along with anti-rabic treatment have a powerful effect in causing the diminution of the incidence of rabies upon the human beings of a country.

**1.5 CONCLUSIONS.**—This concludes the statistical portion of our memoir and we may, before going on to the experimental portion of

CHART I.  
*Rabies in Great Britain.*

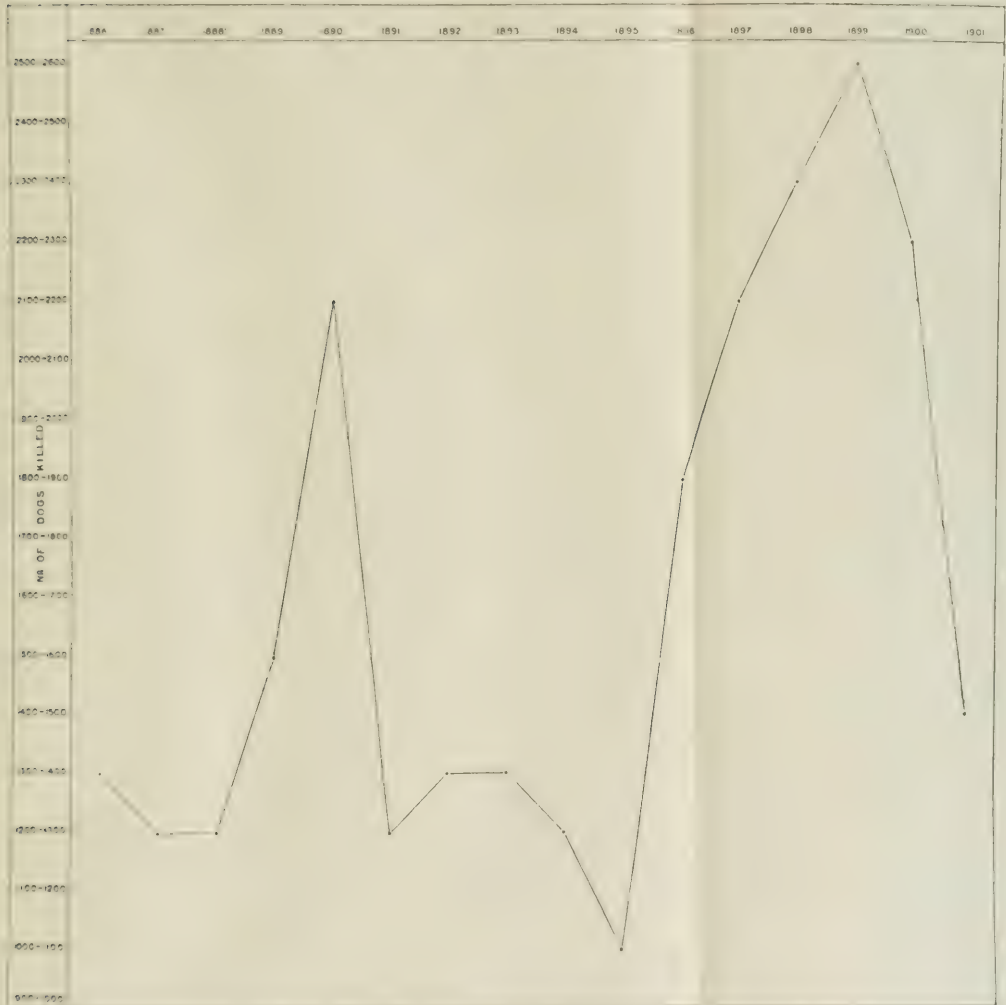


HARVEY W. F. AND ALTON H. W.—An Examination into the Degree of Efficacy of Anti-rabic Treatment.



# CHART II.

*The dogs killed as suspected of rabies (police orders and ownerless) in the German Empire. Laws of 23rd June 1880, 1st May 1894 and § 16 section of "Instructions" of the 12/24th February 1881 and 27th June 1895*  
*Schueder "Die Tollwut &c." 1903 pp. 16. 17.*





it draw our inferences from what has been discussed. Our conclusions are briefly that :—

- (1) The crude death rates from rabies whether amongst treated or untreated should be corrected in accordance with the constitution of the population concerned.
- (2) We should have substitution, or at least inclusion of the total mortality rate in reports, which at present simply give 'failure' rates.
- (3) The current ideas of the mortality occurring amongst the untreated are in need of revision.

TABLE I.

*Death rates among persons treated at Kasauli, 1900-1920.*

Year.						INDIANS.			EUROPEANS.				* Cases extracted.
	Total treated.	Failures.	Per cent.	Total deaths.	Per cent.	Treated.	Deaths.	Per cent.	Treated.	Deaths.	Per cent.	Failures.	
1900-01	321	3	0.9	10	3.1	175	9	5.1	146	1	0.7	..	..
1901-02	543	7	1.3	13	2.4	328	11	3.4	215	2	0.9	1	20
1902-03	584	6	1.0	12	2.1	315	11	3.8	269	1	0.4	..	10
1903-04	612	5	0.8	10	1.6	364	10	2.7	248	..	..	..	50
1904-05	877	7	0.8	12	1.4	570	12	2.1	307	..	..	..	111
1905-06	1,145	7	0.6	21	1.8	803	19	2.4	342	2	0.6	1	117
1906-07	1,308	10	0.7	19	1.5	856	17	2.0	452	2	0.4	1	70
Interval, 9th August, 1907-31st December, 1907	519	1	0.2	5	1.0	373	4	1.1	146	1	0.7	1	..
1908	1,380	5	0.4	26	1.9	1,047	24	2.3	342	2	0.6	..	95
1909	1,937	11	0.6	28	1.4	1,447	25	1.7	490	3	0.6	..	125
1910	2,073	4	0.2	26	1.2	1,719	26	1.5	354	..	..	..	328
1911	2,288	18	0.8	47	2.1	1,971	46	2.3	297	1	0.3	1	473
1912	3,548	21	0.6	46	1.3	3,148	46	1.5	400	..	..	..	390
1913	3,980	29	0.72	48	1.20	3,532	46	1.3	448	2	0.4	1	402
1914	4,585	26	0.57	54	1.18	4,124	53	1.29	461	1	0.22	1	297
1915	5,046	37	0.73	72	1.43	4,724	72	1.52	322	..	..	..	441
1916	5,360	43	0.80	79	1.39	4,982	67	1.34	378	3	0.79	2	393
1917	5,206	44	0.85	68	1.31	4,824	66	1.37	382	2	0.52	2	405
1918	5,690	35	0.62	69	1.21	5,151	67	1.30	520	2	0.38	..	414
1919	6,509	44	0.68	87	1.34	6,067	82	1.35	442	5	1.13	2	440
1920	7,506	35	0.47	65	0.87	6,860	64	0.93	646	1	0.15	..	448
TOTAL	90,996	398	0.45	808	1.33	53,380	777	1.45	7,616	31	0.40	13	5,011

\* In the case of these persons either treatment was not considered necessary, or subsequent events proved that they ran no risk.

TABLE II.

*Number of days before arrival, 1911. Indians and Europeans. Fatal and non-fatal cases.*

NUMBER OF DAYS BEFORE ARRIVAL.

Class.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	Result.
Indian	70	188	193	194	167	216	143	130	119	89	70	56	38	47	39	31	13	15	7	16	17	12	5	9	5	4	3	..	4	12	9	1	3	Non-fatal.																											
European	8	34	35	33	31	33	29	10	11	21	14	10	5	2	12	2	1	1	..	..	3	..	..	..	..	..	..	..	..	1	..	..	Non-fatal.																												
Indian	1	3	7	7	4	4	8	1	2	1	1	..	..	2	..	..	..	..	2	1	..	1	..	..	..	..	..	..	..	..	..	1	Fatal.																												
European	..	..	..	..	..	..	..	..	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Fatal.																												
Percentage Mortality	..	..	2·8	..	..	..	..	..	..	1·8	..	..	..	..	..	..	2·1	..	..	..	..	..	..	0·3	0·3	..	..	..	..	0·3	..	..	..																												

TABLE III.

*Number of wounds. 1911. Indians and Europeans. Fatal and non-fatal cases.*

### NUMBER OF WOUNDS.

[illegible]

TABLE IV.

Nature and number of wounds, 1911. *Indians and Europeans. Fatal and non-fatal cases.*

## I. NON-FATAL CASES.

NATURE *		NUMBER OF WOUNDS.																			
Deep.	Bare.	Uncont. erized.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
+	+	-	19	22	17	16	12	10	5	5	3	3	3	3	1	1	1	1	1	1	1
-	+	-	84	58	37	24	9	11	6	4	3	4	1	1	2	1	1	1	1	1	1
-	+	+	3	65	58	40	41	14	11	6	1	1	3	1	1	1	1	1	1	1	1
+	+	+	43	47	40	49	31	27	17	9	4	11	3	10	1	2	1	4	2	2	2
+	+	+	19	27	16	26	14	19	5	4	3	3	1	1	1	1	1	1	1	1	1
+	+	+	108	124	104	78	57	27	25	8	7	2	5	1	1	1	1	1	1	1	1
+	+	+	53	105	78	76	38	45	19	17	6	17	9	3	5	6	3	4	4	2	2
-	-	-	31	38	23	21	10	5	6	3	1	2	2	2	2	2	2	2	2	2	2

## II. FATAL CASES.

NATURE *		NUMBER OF WOUNDS.																			
Deep.	Bare.	Uncont. erized.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
+	+	-	19	22	17	16	12	10	5	5	3	3	3	3	1	1	1	1	1	1	1
-	+	-	84	58	37	24	9	11	6	4	3	4	1	1	2	1	1	1	1	1	1
-	+	+	3	65	58	40	41	14	11	6	1	1	3	1	1	1	1	1	1	1	1
+	+	+	43	47	40	49	31	27	17	9	4	11	3	10	1	2	1	4	2	2	2
+	+	+	19	27	16	26	14	19	5	4	3	3	1	1	1	1	1	1	1	1	1
+	+	+	108	124	104	78	57	27	25	8	7	2	5	1	1	1	1	1	1	1	1
+	+	+	53	105	78	76	38	45	19	17	6	17	9	3	5	6	3	4	4	2	2
-	-	-	31	38	23	21	10	5	6	3	1	2	2	2	2	2	2	2	2	2	2

\* The presence of a + under the loadings means that this character was present. The presence of a - means that the opposite condition to that signified by the heading existed.

TABLE V.

*Nature and locality of wounds. 1911. Indians and Europeans. Fatal and non-fatal cases.*

## I. NON-FATAL CASES.

NATURE.*			LOCALITY† OF WOUNDS.														
Deep.	Bare.	Uncaut- erized.	1	2	3	4	1 2	1 3	1 4	2 3	2 4	3 4	12 3	12 4	13 4	23 4	12 34
+	-	-	73	12	18	..	..	5	..	1	..	..	..	..	..	..	..
-	-	-	109	5	98	15	..	10	..	2	..	1	1	..	..	..	..
-	-	+	176	31	33	..	1	3	..	2	..	..	..	..	..	..	..
+	-	-	150	2	95	16	..	21	1	7	1	6	1	..	2	1	..
+	-	-	106	13	18	..	1	6	..	1	..	..	..	..	..	..	..
-	-	+	207	14	261	27	1	20	1	4	3	7	1	..	1	1	..
+	-	+	241	10	128	27	3	45	2	10	..	14	2	2	9	2	..
-	-	-	101	15	22	..	..	1	..	..	..	..	1	..	..	..	..

## II. FATAL CASES.

NATURE.*			LOCALITY† OF WOUNDS.															
Deep.	Bare.	Uncaut- erized.	1	2	3	4	1 2	1 3	1 4	2 3	2 4	3 4	12 3	12 4	13 4	23 4	12 34	
-		-	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	
-		-	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
-		+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
+			2	..	2	3	..	..	1	..	..	1	..	..	..	..	1	
+		-	1	..	1	..	..	..	..	..	..	..	..	..	..	..	..	
-		-	3	..	3	..	..	1	..	..	..	1	..	..	..	..	..	
+	+	+	6	..	8	6	..	3	..	..	..	1	1	..	..	..	..	
-		-	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	

\*The presence of a + under the headings means that this character was present. The presence of a - means that the opposite condition to that signified by the heading existed.

†1 = lower extremity; 2 = trunk; 3 = upper extremity; 4 = head and neck;  $\frac{1}{2}$  = lower extremity and trunk, and so with other combinations of these localities.

TABLE VI.

*Number and locality of wounds, 1911. Indians and Europeans. Fatal and non-fatal cases.*

## L. NON FATAL CASES.

Locality.*	NUMBER OF WOUNDS.																			
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	6	21	27	28	12	22	18	21	21	21	21	21	21	21	21	21	21	21	21	21
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

\* 1, lower extremity; 2, trunk; 3, upper extremity; 4, head and neck; 12, lower extremity; and trunk and so with other combinations of these localities.

TABLE VI.—*contd.**Number and locality of wounds, 1911. Indians and Europeans. Fatal and non-fatal cases.—contd.*

## II. FATAL CASES.

## NUMBER OF WOUNDS.

Locality.*	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	..	..	..	..	..	..	1	1	..	1	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
2	..	2	3	1	1	..	1	1	..	1	1	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3	..	..	..	..	..	..	..	..	2	1	1	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	..	..	3	3	3	1	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
12	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
13	..	..	..	..	..	1	1	1	1	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
14	..	..	..	..	..	..	..	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
23	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
24	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
34	..	..	1	1	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
12	..	..	..	..	..	..	..	..	..	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
12	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
13	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
23	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
12	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
34	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

\* 1 = lower extremity; 2 = trunk; 3 = upper extremity; 4 = head and neck; 12 = lower extremity and trunk, and so with other combinations of these localities.





TABLE VIII.

*Showing the incubation period of rabies in Man (Bauer).*

Number of days.	Percentage.
1 to 10	8.24
20 to 30	28.34
40 to 50	21.18
60 to 70	15.30
80 to 90	9.22
100 to 149	7.65
150 to 199	5.60
200 to 249	0.98
250 to 339	2.35
365 to 450	1.18

TABLE IX.

*Showing incubation periods in treated and untreated cases.*

No. of days incubation.	TREATED.			UNTREATED.	
	Hungary Hungary	Paris.	Kassath.	Hungary Hungary.	Various sources Nitsch.
1 to 30	36	67	46	24	14
31 to 90	44	90	65	36	38
61 to 90	15	13	14	23	17
91 to 120	2	5	5	8	13
121 to 150	2	9	0	2	5
151 to 180	0	6	0	2	5
181 to 210	2	1	1	1	1
211 to 240	1	2	0	1	2
241 to 270	0	1	2	0	2
271 to 300	0	1		2	0
301 to 330	0	0		0	0
331 to 360	1	0		0	0
361 to 390	1	0		1	1
391 to 420	0	0		1	0
507	0	0			1
580	0	0			1
646	1	0			
700		1			
616		1			
1851		1			

TABLE X.

*Rabies among British troops in India, Indian troops and Indian prisoners,  
from 1871 to 1918.*

Year.	BRITISH ARMY.						INDIAN ARMY.		INDIAN PRIS- ONERS.		TOTALS.
	MEN.		WOMEN.		CHILDREN.		MEN.		MEN.		
	Average annual strength.	Cases.	Average annual strength.	Cases.	Average annual strength.	Cases.	Average annual strength.	Cases.	Average annual strength.	Cases.	
1871	56,784	5	6,384	0	10,700	3	..	..	..	..	8
1872	58,694	2	6,650	0	11,657	0	..	..	..	..	2
1873	58,816	1	6,626	0	11,860	2	..	..	..	..	3
1874	59,253	2	6,627	0	12,393	1	..	..	..	..	3
1875	59,368	2	6,335	0	12,359	1	..	..	..	..	3
1876	58,506	2	6,050	0	11,882	0	..	..	..	..	2
1877	58,032	3	5,644	0	11,266	0	113,966	6	110,147	1	5
1878	56,664	1	5,170	0	10,423	0	117,273	6	127,914	5	12
1879	57,810	0	4,640	0	8,993	0	121,107	3	117,680	1	4
1880	60,034	4	4,134	0	7,596	0	126,385	2	106,778	1	6
1881	58,411	1	3,741	0	6,548	1	114,612	1	100,856	2	5
1882	57,198	1	3,539	0	6,208	1	114,894	2	94,059	0	4
1883	55,454	4	3,290	0	6,015	0	114,830	5	88,178	0	9
1884	55,349	4	3,371	0	6,453	0	114,827	5	85,500	2	11
1885	57,116	3	3,419	0	6,632	1	115,486	2	82,644	0	6
1886	61,750	3	3,391	0	6,552	1	106,010	6	84,909	1	11
1887	63,924	3	3,242	0	6,333	0	105,254	3	79,471	1	7
1888	68,594	0	3,228	0	6,168	3	124,822	3	91,445	3	9
1889	69,017	1	3,166	0	6,041	0	128,642	1	96,121	1	3
1890	67,809	2	3,130	0	5,912	0	127,746	1	96,610	3	6
1891	66,956	3	3,137	0	5,886	0	128,600	3	101,019	1	7
1892	68,162	1	3,101	0	5,762	1	127,355	0	103,159	6	8
1893	70,063	3	3,135	0	5,662	0	127,091	2	101,737	1	6
1894	71,094	5	3,157	0	5,680	0	127,844	3	101,965	1	9
1895	71,031	3	3,194	0	5,699	0	129,211	1	106,337	1	5
1896	70,484	3	3,254	0	5,790	1	128,187	0	110,090	1	5
1897	64,531	2	3,203	0	5,744	1	118,486	0	118,107	1	4
1898	65,397	2	3,118	0	5,592	0	121,318	3	111,344	1	6
1899	67,697	1	3,093	0	5,500	0	127,019	2	110,016	0	3
1900	60,553	3	2,908	0	5,376	0	123,463	0	121,811	4	7
1901	60,838	2	2,729	0	5,069	0	122,806	7	117,203	0	9
1902	60,540	2	2,555	0	4,709	0	124,231	0	114,334	3	5
1903	69,612	1	2,891	0	4,677	1	124,660	4	101,717	0	6
1904	70,613	2	3,226	0	4,964	0	124,055	1	104,325	1	4
1905	70,994	2	3,375	0	5,154	0	123,434	6	106,265	0	8
1906	70,193	2	3,431	0	5,322	1	127,853	1	110,082	0	4
1907	69,332	0	3,496	0	5,379	0	126,392	1	107,675	1	2
1908	68,522	2	3,696	0	5,819	0	126,975	2	115,403	0	4
1909	71,556	2	3,913	0	6,201	0	131,627	2	112,249	4	8
1910	72,491	2	4,137	0	6,678	0	130,937	1	110,455	1	4
1911	72,371	1	4,248	0	7,056	0	130,441	0	109,099	1	2
1912	71,001	0	4,147	0	7,046	1	131,644	0	103,906	0	1
1913	70,755	2	4,123	0	7,006	0	131,512	0	108,286	1	3
1914	50,581	1	3,772	0	6,465	0	124,894	2	114,113	0	3
1915	44,891	1	1,570	0	2,925	0	118,823	7	123,169	2	10
1916	60,737	2	1,554	0	2,830	0	139,076	0	126,642	4	6
1917	80,825	1	1,463	0	2,629	0	191,242	4	118,121	0	5
1918	87,982	0	1,481	0	2,624	0	341,458	3	120,215	2	5

TABLE XI.

*Statistics of annual mortality in Prussia for a series of years.*

Authority.	Year.	Number Bitten.	Deaths.	Percentage.	Notes.
1. Schueder Die Tollwut in Deutsch- land und ihre Be-K am p- fung, p. 3.	1866-67 ..	..	11	..	
	1867-68 ..	..	7	..	
	1868-69 ..	..	9	..	
	1869-70 ..	..	5	..	
	1870-71 ..	..	12	..	
	1871-72 ..	..	24	..	
	1872-73 ..	..	23	..	
	1873-74 ..	..	13	..	
	1874-75 ..	..	12	..	
	1875-76 ..	..	12	..	
	1876-77 ..	..	8	..	
	1877 ..	..	13	..	
	1878 ..	..	15	..	
	1879 ..	..	10	..	
	1880 ..	..	15	..	
	1881 ..	..	12	..	
	1882 ..	..	5	..	
	1883 ..	..	3	..	
	1884 ..	..	3	..	
	1885 ..	..	6	..	
	1886 ..	..	4	..	
	1887 ..	..	1	..	
	1888 ..	..	1	..	
	1889 ..	..	3	..	
	1890 ..	..	2	..	
2. Kirschner Klin. Jahrb. 10-2-1902, p. 177.	1891 ..	78	4	5.13	
	1892 ..	72	4	5.56	
	1893 ..	60	4	6.67	
	1894 ..	92	2	2.17	
	1895 ..	66	2	3.03	
	1896 ..	128	4	3.13	
	1897 ..	161	5	3.11	
	1898 ..	263	9	3.42	
	1899 ..	303	3	0.99	
	1900 ..	233	0	0	
	1901 ..	187	1	0.54	
					The figures for Prussia are extracted from figures for all Germany, when not directly given. Pasteur Institute, Paris founded 1888.
3. Dardart Klin. Jahrb. 21-1-1909, p. 1.	1902 ..	250	6	0.24	
	1903 ..	307	6	0.20	
	1904 ..	365	8	0.22	
	1905 ..	368	12	0.33	
	1906 ..	373	4	0.11	
	1907 ..	465	4	0.10	
					First case treated in Berlin 1898. Berlin Institute, founded 18-7-98.

TABLE XII.

*Showing the results obtained at different Institutes at one time or another.**From : -Babes ' Traité de la Rage. 1912, p. 591.*

Institute.	Years.	No. of cases treated.	Deaths.	Percentage.
Tunis .. .. .	Opened 1906	2,490	9	0·36
Paris .. .. .	1886-1905	29,937	129	0·41
Buda Pesth .. .. .	1890-1905	32,508	126	0·46
Algiers .. .. .	1886-1895	4,194	59	1·20
Do. .. .. .	1894-1905	5,395	19	0·35
Marseilles .. .. .	1893-1903	3,563	13	0·36
Lyons .. .. .	1900-1906	5,374	6	0·11
Do. .. .. .	1898-1902	1,415	6	0·92
Berlin .. .. .	1906-1907	312	3	0·99
Vienna .. .. .	1892-1903	1,937	13	0·68
Kharkoff .. .. .	1892-1901	9,740	56	0·59
Lille .. .. .	1895-1902	1,807	4	0·22
Pernambuco .. .. .	1889-1903	486	1	0·20
Sofia .. .. .	1902-1904	1,081	6	0·55
Rome .. .. .	1889-1902	1,940	7	0·37
Naples .. .. .	1886-1906	8,446	45	0·53
Faenza .. .. .	1898-1902	779	1	0·12
Milan .. .. .	1889-1903	2,942	24	0·83
Florence .. .. .	1889-1901	1,254	2	0·15
Jassy .. .. .	1891-1905	3,038	5	0·16
Cairo .. .. .	1889-1901	375	1	0·26
New York .. .. .	1890-1901	1,608	10	0·62
Bucharest .. .. .	1888-1905	9,250	11	0·13
Lisbon .. .. .	1893-1905	8,844	44	0·50
Constantinople .. .. .	1900-1905	4,100	15	0·50
St. Petersburg .. .. .	1901	592	1	0·16
Kerson .. .. .	1901-1902	600	5	0·83
Warsaw .. .. .	1900	923	9	0·97
Do. .. .. .	1892	324	11	3·39
Kasauli .. .. .	1905-1906	1,149	7	0·61
Sassari .. .. .	1900-1908	1,053	2	0·19
Turin .. .. .	1886-1895	2,254	22	0·86
Palermo .. .. .	1887-1895	2,221	9	0·40
Moscow .. .. .	1892	107	9	8·40
Odessa .. .. .	1892	324	11	3·39
Samara .. .. .	1892	53	3	7·67
Saigon .. .. .	1886-1895	110	2	1·82

TABLE XIII.

*Showing statistics and death rates for the Paris Institute, from 1886 to 1919*  
*From Babes' *Traité de la Rage*, p. 591, supplemented from the *Annales**  
*de l'Institut Pasteur.*

Year	Persons treated	Deaths	Mortality %
1886	2,671	25	0.94
1887	1,770	14	0.79
1888	1,662	9	0.55
1889	1,830	7	0.38
1890	1,540	5	0.32
1891	1,559	4	0.25
1892	1,790	4	0.22
1893	1,648	6	0.36
1894	1,387	7	0.50
1895	1,520	5	0.33
1896	1,308	4	0.30
1897	1,521	6	0.39
1898	1,465	3	0.20
1899	1,614	4	0.25
1900	1,420	4	0.28
1901	1,321	5	0.38
1902	1,105	2	0.18
1903	628	2	0.32
1904	755	3	0.39
1905	727	3	0.41
1906	772	1	0.13
1907	786	3	0.38
1908	524	1	0.19
1909	467	1	0.21
1910	461	0	0.0
1911	341	1	0.29
1912	395	0	0.0
1913	330	0	0.0
1914	373	0	0.0
1915	654	1	0.15
1916	1,388	3	0.21
1917	1,543	4	0.26
1918	1,803	3	0.16
1919	1,813	3	0.16

TABLE XIV.

*Showing the effect of restrictive measures on the occurrence of rabies in dogs  
Department of the Seine in France. 'Lancet,' 19-11-10, p. 1504.*

Year.	DEPARTMENT OF THE SEINE.		OTHER DEPARTMENTS. MORTALITY RATE.	
	Rabid dogs.	Dogs captured.	Rabid dogs.	Paris Institute.
1899 ..	....	9,691	....	0·25
1900 ..	807	12,000	1,964	0·28
1901 ..	845	16,298	1,872	0·38
1902 ..	474	16,856	1,852	0·18
1903 ..	179	14,665	2,184	0·32
1904 ..	172	15,251	2,221	0·39
1905 ..	120	....	2,248	0·41
1906 ..	74	....	1,971	0·13
1907 ..	43	....	1,849	0·38
1908 ..	37	....	1,445	0·19
1909 ..	13	....	1,450	0·21
1910 ..	....	....	....	0·00

TABLE XV.

*Showing numbers treated and deaths according to period of treatment in  
Constantinople 1901 to 1908 (Remlinger).*

Year.	No. of persons treated.	Died during treatment.	Died less than 15 days after treatment.	Died more than 15 days after treatment.	TOTALS
1901 ..	572	5	1	1	7
1902 ..	750	5	4	4	13
1903 ..	894	9	1	3	13
1904 ..	1,075	7	1	3	11
1905 ..	809	5	4	3	12
1906 ..	873	12	5	1	18
1907 ..	857	5	2	1	8
1908 ..	978	12	2	3	17
TOTALS ..	6,808	60 (0·88%)	20 (0·29%)	19 (0·27 %)	99 (1·45%)

TABLE XVIII.

*Showing the periods of incubation, interval to death, and duration of symptoms of Street Virus and Fixed Virus in rabbits inoculated subdurally.*

NUMBER OF DAYS.																																																																			
Period.	Virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	43	44	45	46	49	51	58	59	60	61	62	63	64	NOTES.													
1. Incubation as judged from onset of paralysis.	Street	..	..	..	..	1	3	9	14	31	35	103	96	76	65	57	35	24	16	14	13	7	7	3	3	3	8	3	.	3	3	3	..	1	5	1	..	..	1	..	1	1	1	..	1	..	1	1	..	..	..	Kasauli Insti- tute.															
2. Interval death.	to Street	..	..	..	..	..	3	1	5	11	18	47	71	76	81	81	62	49	29	22	22	8	7	7	8	4	4	7	2	2	3	3	4	1	2	1	2	..	1	..	..	..	1	1	1	..	..	1	..	..	1	1	Kasauli Insti- tute.														
3. Incubation ..	Street	..	..	..	..	..	3	7	6	4	10	21	26	45	35	33	17	18	7	7	6	10	5	5	2	1	4	3	..	2	..	2	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Calabrese In- Past., 1896														
4. Incubation as judged from onset of symptoms of staggering.	Fixed	..	..	..	..	5	116	390	132	5	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Kasauli Insti- tute.															
5. Interval death.	to Fixed	..	..	..	..	..	..	..	..	77	233	237	93	10	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Kasauli Insti- tute.														
6. Duration of symptoms.	Street	79	122	122	38	16	12	6	4	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Kasauli Insti- tute.															
7. Duration of symptoms.	Fixed	3	69	165	121	37	5	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Kasauli Insti- tute.														



TABLE XVI.

*Showing dates of arrival patients. Kasauli.*

Arrival at Institute.	1901	1902	1903	1904	1905	1906	1907	1908	TOTALS
From 1st to 5th day .	41	131	152	190	171	174	243	222	1,324
From 5th to 10th day .	148	220	279	275	232	246	225	280	1,914
From 10th to 15th day .	130	183	247	269	180	236	175	224	1,653
From 15th to 20th day .	117	96	121	188	112	90	106	128	958
From 20th to 25th day .	88	62	53	73	56	60	48	55	495
From 25th to 30th day .	28	31	24	42	39	22	20	20	226
From 30th to 35th day .	3	7	12	15	6	20	13	12	88
From 35th to 40th day .	7	7	3	10	6	12	7	7	59
From 40th to 45th day .	1	8	2	6	1	4	7	9	38
After the 45th day .	0	5	1	7	6	9	13	12	53
TOTALS .	572	750	894	1,075	809	873	857	978	6,808
MEAN DATE OF ARRIVAL	13	12	12	13	13	13	12	14	..

TABLE XVII.

*Showing the results of an enquiry into death rates amongst treated and untreated at Kasauli.*

	TREATED.				UNTREATED.					
	Alive.	Dead.	Percentage.		TOTALS.			PERCENTAGE.		
					Official.	Patients' statement.	Alive.	Dead.	Official.	Patients' statement.
Total.	599	599	9	1.2	1,119	1,530	999	120	10.7	8.4

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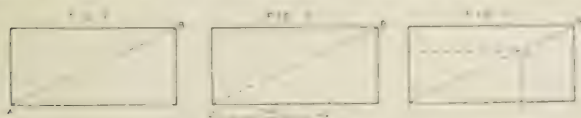
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## INDEX OF AUTHORS.

### A

	Page
ACTON, H. W. On the Behaviour of <i>Paramecium Caudatum</i> towards the Cinchona Alkaloids .. .. .	339
ACTON, H. W. Melanotic Growths. Researches on the alterations in the Melanoblastic layer of vertebrates, to explain the structure and origin of these growths. Sections I.—III.	464
ACTON, H. W. <i>See</i> HARVEY, W. F.	
ADIE, H. A. A preliminary note on the Development of the Leishman-Donovan Parasite in Spleen Juice and in the Alimentary Tract of <i>Cimex Lectularius</i> .. .. .	255
ADIE, H. A. Telegram announcing finding Leishman-Donovan bodies in Salivary glands and ducts of <i>Cimex rotundatus</i> ..	v
AVARI, C. R. <i>See</i> TURKHUD, D. A.	
AWATI, P. R. Some Notes on <i>Conorhinus Rubrofasciatus</i> (de Geer) .. .. .	371

### C

CAIUS, J. F. <i>See</i> MHASKAR, K. S.	
CHRISTOPHERS, S. R., and CRAGG, F. W. On the So-called 'Penis' of the Bed-bug ( <i>Cimex Lectularius</i> , L.) and on the homologies generally of the male and the female Genitalia of this Insect ..	445
CORNWALL, J. W. and LA FRENAYS, H. M. A contribution to the Study of Kala-Azar (V.) .. .. .	533
CORNWALL, J. W. Note on the Histo-Pathology of a Non-ulcerated Oriental Sore .. .. .	545
CRAGG, F. W. Geographical Distribution of the Indian Rat Fleas as a Factor in the Epidemiology of Plague: Preliminary Observations .. .. .	374
CRAGG, F. W. <i>See</i> CHRISTOPHERS, S. R.	

### E

EDITORIAL. Note on bodies found by Mrs. Adie in Salivary glands by Bed-bug .. .. .	v
------------------------------------------------------------------------------------	---

	Page
<b>G</b>	
GILL, C. A. Relapsing fever in the Punjab .. ..	747
GUPTA, BIRAJ M. DAS. A Note on Some Cultural Phases of Leishmania Donovanii .. .. .	809

<b>H</b>	
HARVEY, W. F. Bacteriological and Laboratory Technique. Section II.—Media .. .. .	66
HARVEY, W. F. Bacteriological and Laboratory Technique— Section III .. .. .	261
HARVEY, W. F. and IYENGAR, K. R. K. Desiccated Nutrient Media .. .. .	364
HARVEY, W. F. Bacteriological and Laboratory Technique. Section IV .. .. .	405
HARVEY, W. F. Bacteriological and Laboratory Technique. Section V .. .. .	692
HARVEY, W. F. and IYENGAR, K. R. K. Virulence of Micro- organisms and its dependence on the culture medium ..	726
HARVEY, W. F. and IYENGAR, K. R. K. Virulence of the organism as a factor in the efficacy of Prophylactic vaccines ..	730
HARVEY, W. F. and IYENGAR, K. R. K. On the Immunizing Properties of Allied organisms and non-specific organisms ..	736
HARVEY, W. F. Immunization (1) Response of Immunized animals to a small dose of Antigen administered at a long interval after first immunization .. .. .	740
HARVEY, W. F.; and ACTON, H. W. An Examination into the Degree of efficacy of anti-rabic treatment .. .. .	852
HINGSTON, R. W. G. Physiological changes at High Altitudes and their relation to mountain-sickness .. .. .	173
HODGKINSON, W. H. and HUTCHINSON, C. M. Report on Electrolytic Chlorogen (E. C.) .. .. .	586
HUTCHINSON, C. M. <i>See</i> HODGKINSON, W. H.	

<b>I</b>	
IYENGAR, K. R. K. <i>See</i> HARVEY, W. F.	
IYENGAR, K. R. K. Studies in the Value of the Wassermann Test. No. IV—Significance and Value of a Positive Wasser- mann Reaction in Tuberculosis .. .. .	369

	Page
IVENGAR, M. O. T. A Note on grappling Tail-Hooks in Anophe- line Larvæ .. .. .	630

**L**

LA FREN AIS, H. M. See PATTON, W. S.	
LA FREN AIS, H. M. See CORNWALL, J. W.	
LANE, CLAYTON. Some Bursate Nematodes from Indian and African Elephants .. .. .	163

**M**

MACKIE, F. P. Visceral Infections due to the higher Fungi ..	781
MALONE, R. H. The Production of B. Influenzae Vaccine on a large scale .. .. .	56
MALONE, R. H. A Note on the large scale production of Pneumococcus Vaccine .. .. .	63
MALONE, R. H. A Bacteriological Investigation of Influenza carried out under the Indian Research Fund Association. Part II. Influenza in Bombay, July, 1919-June, 1920 ..	210
MALONE, R. H. The demonstration of the capsules of the Pneu- mococcus by a modification of Benian's relief stain ..	804
McVAIL, J. BORLAND. Preliminary note on Septic tank latrines in relation to Hookworm disease .. .. .	806
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. X—Betanaphthol .. .. .	33
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XI—Extractum Filicis Liqui- dum .. .. .	191
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Thera- peutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XII—Kousso .. .. .	198
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XIII—Karnam .. .. .	202

	Page
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XIV—Pulvis Arecae ..	206
MHASKAR, K. S. and CAIUS, J. F. The Correlation between the Chemical Composition of Anthelmintics and their Therapeutic values in connection with the Hookworm Inquiry in the Madras Presidency. No. XV—Salicylic Acid, and its derivatives .. .. .	787
MHASKAR, K. S. Delayed cures in the treatment of Hookworm Infection .. .. .	799

## N

NAPIER, L. E. A new Serum test for Kala-Azar ..	830
-------------------------------------------------	-----

## P

PATTON, W. S. Studies on the Flagellates of the Genera Herpetomonas, Crithidia and Rhynchoidomonas No. 7—Some Miscellaneous Notes on Insect Flagellates ..	230
PATTON, W. S., LA FRENAIS, H. M. and RAO, SUNDARA. Studies on the Flagellates of the Genera Herpetomonas, Crithidia Rhynchoidomonas No. 8.—Note on the Behaviour of Herpetomonas Tropica Wright, the Parasite of Cutaneous Herpetomoniasis (Oriental Sore), in the Bed-bug, Cimex Hemiptera Fabricus .. .. .	240
PATTON, W. S., LA FRENAIS, H. M. and RAO, SUNDARA. Studies on the Flagellates of the Genera Herpetomonas, Crithidia Rhynchoidomonas No. 9.—Note on the Behaviour of Herpetomonas Donovan, Laveran and Mesnil in the Bed-bug, Cimex Hemiptera Fabricus .. .. .	252
PATTON, W. S. Some Reflections on the Kala-Azar and Oriental Sore Problems .. .. .	496
PATTON, W. S. Some Notes on Indian Calliphorinæ. Part II.—Lucilia Argyricephala Macq. (Serenissima Fabr.). The Common Indian Bazaar Green Bottle, whose Larvæ occasionally cause cutaneous myiasis in animals and Lucilia Craggii Sp. Nov. One of the Common Blow Flies of Indian Hill Stations .. .. .	548

	Page
PATTON, W. S. Some Notes on Indian Calliphorinae. Part III.— Chrysomyia Megacephala Fabr. (Dux Esch.). The Common Indian Blue Bottle, whose Larvæ occasionally cause cutaneous myiasis in animals and Chrysomyia Nigriceps Sp. Nov. The Common Blue Bottle of the Nilgiris .. ..	555
PATTON, W. S. Some Notes on Indian Calliphorinae. Part IV.—Chrysomyia Albiceps Wied. (Putoria Wied Rufifacies Froggatt.) One of the Australian Sheep Maggot Flies and Chrysomyia Villeneuvei Sp. Nov. .. ..	561
PATTON, W. S. Some Notes on Indian Calliphorinae. Part V.— Lucilia Pulchra Wied. (Ruficornis Macq.) A Larviparous Calliphorine and Lucilia Ballardii Sp. Nov. A Common South Indian Blow Fly .. ..	570
PATTON, W. S. Some Notes on Indian Calliphorinae. Part VI.— How to recognise the Indian Myiasis producing flies and their larvæ together with some notes on how to breed them and study their habits .. ..	635
PATTON, W. S. Some Notes on Indian Calliphorinae. Part VII.— Additional cases of Myiasis caused by the Larvæ of Chryso- myia Bezziana Vill. together with some notes on the Diptera which cause myiasis in man and animals .. ..	654
PATTON, W. S. Notes on Some Indian Aphiochaetae. Aphiochaetae Xanthina Speiser (Repicta Schmitz. Circumsetosa De Meijere, Ferruginea Brunetti). Whose larvæ cause cutaneous and Intestinal Myiasis in man and animals and Aphiochaeta Rutipes Meigen whose Larvæ occasionally cause cutaneous myiasis in animals .. ..	683

## R

RAMA KRISHNAN, S. The Reliability of Gate and Papacostas' Formol-Gel Test for Syphilis as compared with the Wassermann Reaction .. ..	620
RAO, SUNDARA. See PATTON, W. S.	

## S

SHORTT, H. E. Review of the Position of the Genus Homocystidium (Castellani and Wiley, 1904), with a description of two new species .. ..	744
-------------------------------------------------------------------------------------------------------------------------------------------	-----

	Page
SHORTT, H. E. Notes on two Hæmogregarines of Persian Lizards .. .. .	827
SINTON, J. A. Some cases of Myiasis in India and Persia, with a Description of the Larvæ causing the Lesions ..	132
SINTON, J. A. Infection with Nuttallia Ninense among Hedgehogs in the North-West Frontier Province, India ..	359
SINTON, J. A. Entomological Notes on Field Service in Waziristan .. .. .	575
SOPARKAR, M. B. The Cercaria of <i>Schistosomum Spindalis</i> (Montgomery) .. .. .	1
SOPARKAR, M. B. Notes on Some Furcocercous Cercariæ from Bombay .. .. .	23
SPRAWSON, C. A. The Heart in Beri-Beri and the evidence of the Differential Stethoscope .. .. .	625

## T

TURKHUD, D. A. and AVARI, C. R. Gaté—Papacostas' Reaction in Leprosy .. .. .	850
------------------------------------------------------------------------------	-----

# INDEX OF SUBJECTS.

## A

	Page
Anopheline Larvae. Grappling tail hooks in—	630
Anthelmintics. Their Chemical Composition and Therapeutic Values	33, 191, 198, 202, 206, 787
Antigen. Immunization or response of Immunized animals to a small dose of—	740
Anti-rabic Treatment. Efficacy of—	852
Aphiochaeta Rufipes Meigen	683
Aphiochaeta Xanthina Speiser	683
Arecas. Pulvis. in Hookworm disease	206
Authors. Instruction to—	890

## B

Bacteriological and Laboratory Technique	66, 261, 405, 692
Bed-bug (Cimex Lectularius L.). Penis of—	115
Bed-bug. Homologies of the male and female genitalia of—	115
Bed-bug. Note on bodies found by Mrs. Adie in Salivary glands of—	v
Beri-Beri. Heart in and the evidence of Differential Stethoscope	625
Betanaphthol in Hookworm Disease	33
Bombay. Influenza in	210
Bursate Nematodes from Indian and African Elephants	163

## C

Calliphorinae, Indian. Some notes on—	518, 555, 561, 570, 635, 654
Caudatum. Paramecium. Behaviour of towards the Cinchona Alkaloids	339
Cercaria of Schistosomum Spindalis	1
Cercaria. Some Furcocercous—from Bombay	23
Chlorogen, Electrolytic (E. C.)	586

	Page
Chrysomyia Albiceps Wied .. .. .	562
Chrysomyia Bezziana Vill .. .. .	654
Chrysomyia Megacephala Fabr. .. .. .	556
Chrysomyia Nigriceps .. .. .	558
Chrysomyia Villeneuvei .. .. .	567
Cinchona Alkaloids. Behaviour of Paramecium Caudatum towards— .. .. .	339
Cinchona Alkaloids. Toxicity of .. .. .	349
Congress, Indian Science. Notice .. .. .	399, 401
Conorhinus Rubrofasciatus (de Geer) .. .. .	371
Crithidia Rhynchodomonas. Flagellates of .. .. .	230, 240, 252
Culture Medium. Virulence of Micro-organisms dependent on .. .. .	726

**D**

Diptera which cause myiasis in man and animals .. .. .	654
--------------------------------------------------------	-----

**E**

Electrolytic Chlorogen (E. C.) .. .. .	586
Elephants. Bursate Nematodes from Indian and African .. .. .	163

**F**

Field Service in Waziristan. Entomological Notes on— .. .. .	575
Filicis Liquidum, Extractum—in Hookworm Disease .. .. .	191
Flagellates, Insect .. .. .	230
Flagellates of the Genera Herpetomonas, Crithidia and Rhynchodomonas. Studies on the— .. .. .	230, 240, 252
Formol-Gel Test for Syphilis as compared with Wassermann Reaction .. .. .	620
Fungi, Higher—due to Visceral Infections .. .. .	781

**G**

Gaté and Papacostas' Formol-Gel Test. Reliability of—for Syphilis as compared with the Wassermann Reaction .. .. .	620
Gaté—Papacostas' Reaction in leprosy .. .. .	850
Genitalia of male and female Bed-bug. Homologies of— .. .. .	445
Genus Hæmocystidium. Review of the position of— .. .. .	814

# Index of Subjects.

903

Page

## H

Hæmogregarines of Persian Lizards .. .. .	827
Hæmocystidium. Position of the Genus .. .. .	814
Hedgehogs. Infection with <i>Nuttallia Ninense</i> among—in the North-West Frontier Province, India .. .. .	359
Herpetomonas <i>Uraggii</i> .. .. .	232
Herpetomonas Donovan. Laveran and Mensil. Behaviour of— in the Bed-bug <i>Cimex Hemiptera</i> Fabricus .. .. .	252
Herpetomonas, Genera. Flagellates of .. .. .	230, 240, 252
Herpetomonas <i>Mirabilis</i> Roubaud .. .. .	233
Herpetomonas <i>Muscae domesticæ</i> Burnett .. .. .	236
Herpetomonas <i>Sarcophagæ</i> Prowazek .. .. .	238
Herpetomonas Tropica Wright, the Parasite of cutaneous Herpetomoniasis (oriental sore). Behaviour of—in the Bed- bug, <i>Cimex Hemiptera</i> Fabricus .. .. .	240
Hookworm Disease and Septic tank latrines .. .. .	806
Hookworm Infection. Delayed cures in the treatment of .. .. .	799
Hookworm Inquiry in the Madras Presidency 33, 191, 198, 202, 206, .. .. .	787

## I

Immunization .. .. .	740
Infections visceral—due to the Higher Fungi .. .. .	781
Influenza. Bacteriological investigation of—in Bombay .. .. .	210
Influenza Vaccine. Production of—on a large scale .. .. .	56
Instructions to Authors .. .. .	890

## K

Kala-Azar. Contribution to the study of .. .. .	533
Kala-Azar inquiry of the Indian Research Fund Association— An announcement .. .. .	v
Kala-Azar. New Serum test for .. .. .	830
Kala-Azar Problem .. .. .	498
Kala-Azar. Reflections on .. .. .	496
Kamala in Hookworm Disease .. .. .	202
Kousso in Hookworm Disease .. .. .	198

## L

Larvæ causing the Lesions .. .. .	132
Leishman-Donovan bodies in Salivary glands and ducts of <i>Cimex rotundatus</i> .. .. .	v

	Page
Leishman-Donovan Parasite. Development in Spleen Juice and in the Alimentary tract of <i>Cimex Lectularius</i> ..	255
Leishmania-Donovani. Some Cultural phases of ..	809
Leprosy. Gaté—Papacostas' Reaction in ..	850
Lizards, Persian. Two <i>Hæmogregarines</i> of ..	827
<i>Lucilia Argyricephala</i> Macq ..	549
<i>Lucilia Ballardii</i> ..	572
<i>Lucilia Craggii</i> Sp. Nov. ..	552
<i>Lucilia Pulchra</i> Wied ..	571
<i>Lucilia Serricata</i> ..	150

### M

Madras Presidency. Hookworm Inquiry in -33, 191, 198, 202, 206, 787	
Malarial fever. Treatment of ..	355
Media, Desiccated Nutrient ..	364
Melanin ..	473
Melanoblasts ..	467
Melanomata, Benign ..	477
Melanomata, Innocent ..	478
Melanomata, Malignant ..	485
Melanotic Growths ..	464
Melanotic malignant growths. Structure of ..	488
Micro-organisms. Virulence of—and its dependence on the culture medium ..	726
Mountain-sickness. Physiological at High Altitudes and their relation to— ..	174
<i>Musca autumnalis</i> ..	231
<i>Musca bezzii</i> ..	230
Myiasis in India and Persia ..	132
Myiasis—Producing flies, Indian. How to recognise them, breed them and study their habits ..	635

### N

Nematodes from Indian and African Elephants ..	163
Nutrient Media, Desiccated ..	364
Nuttallia Ninense. Infection with—among Hedgehogs in the North-West Frontier Province, India ..	359

## O

Organisms, allied. Immunizing properties of— .. ..	736
Organisms, Non-specific. Immunizing properties of .. ..	736
Organism, virulence of— as a factor in the Efficacy of Prophylactic vaccines .. ..	730
Oriental Sore Problems. Reflections on— .. ..	522
Oriental Sore, Non-ulcerated. Histo-pathology of— .. ..	545

## P

Paramecium caudatum. Behaviour of— towards the Cinchona Alkaloids .. ..	339
Paramecium. Cultivation of .. ..	352
Parasite in the bed-bug—its behaviour .. ..	245
Phlebotomi .. ..	574
Plague. Epidemiology of. Geographical Distribution of Indian Rat Fleas as a factor in the— .. ..	374
Pneumococcus. Demonstration of the capsules of— by a modification of Benian's relief stain .. ..	804
Pneumococcus. Large scale production of— .. ..	63
Prophylactic vaccines. Virulence of the organism as a factor in the efficacy of— .. ..	730
Pulvis Arecae in Hookworm Disease .. ..	206
Pycnosoma Dux. Description of— .. ..	140

## R

Rat Fleas, Indian. Geographical Distribution of .. ..	374
Relapsing fever in the Punjab .. ..	747
Rhynchodomonas, Crithidia. Flagellates of— .. ..	230, 240, 252
Rubrofasciatus (de Geer), Conorhinus .. ..	371

## S

Salicylic Acid and its derivatives in Hookworm Disease .. ..	787
Sarcophaga ruficornis. Description of .. ..	153
Schistosomum Spindalis. Cercaria of .. ..	1
Sepsids. Flagellates of .. ..	238
Septic tank latrines and Hookworm Disease .. ..	66
Sputum. Bacteriological Examination of .. ..	211

	Page
Stethoscope. The Heart in Beri-Beri and the Evidence of Differential— .. .. .	625
Syphilis. Formol-Gel Test for—as compared with the Wassermann Reaction .. .. .	620

**T**

Technique, Bacteriological and Laboratory .. 66, 261, 405,	692
Tuberculosis. Significance and value of a positive Wassermann Reaction in— .. .. .	369

**V**

Vaccine, Pneumococcus. Large scale production of ..	63
Vaccine, B. Influenzæ. Large scale production of ..	56
Vaccine, Prophylactic. Virulence of the organism as a factor in the efficacy of .. .. .	730
Visceral Infections due to the Higher Fungi .. .. .	781

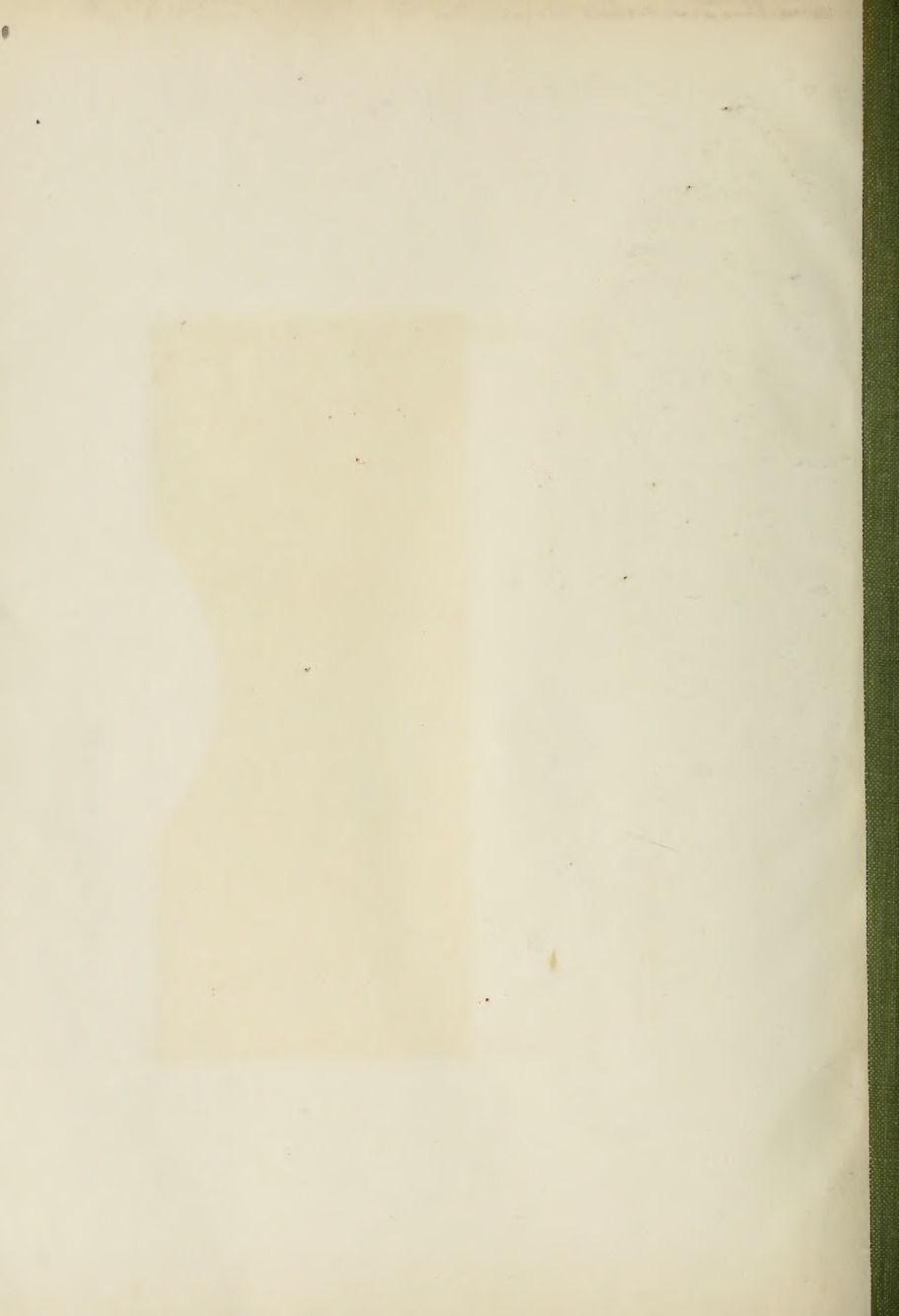
**W**

Wassermann Reaction. Positive—in Tuberculosis .. .. .	369
Wassermann Reaction. Reliability of Gaté Papacostas' Formol-Gel Test for Syphilis as compared with— .. .. .	620
Wassermann Test. Value of .. .. .	369
Waziristan. Entomological Notes on Field Service in— ..	575
Wohlfahrtia Sp. Description of— .. .. .	157









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